WHOLE WHEAT PHYTOCHEMICALS AND POTENTIAL HEALTH BENEFIT

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Neal Okarter

May 2010

WHOLE WHEAT PHYTOCHEMICALS AND POTENTIAL HEALTH BENEFIT

Neal Okarter, Ph. D.

Cornell University 2010

A whole grain consists of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components - the starchy endosperm, germ and bran - are present in the same relative proportions as they exist in the intact caryopsis. Increased consumption of whole grains has been associated with reduced risk of major chronic diseases. Phytochemicals are bioactive non-nutrient plant compounds in fruits, vegetables, whole grains, and other plant foods. Whole grain foods offer a wide range of phytochemicals that are proposed to be responsible for the health benefits of whole grain consumption. The objective of this dissertation was to determine the phytochemical content, antioxidant activity, and antiproliferative activity of whole wheat and its milled fractions, the bran/shorts, germ, and endosperm.

The most studied whole grain phytochemicals are phenolics, compounds with one or more aromatic rings and one or more hydroxyl groups. Phenolics are generally found bound to cell wall polymers and are therefore insoluble upon extraction. The bound fraction contributed 53.8 to 69.7% of the total phenolic content of the six diverse wheat varieties analyzed. The insoluble-bound fraction of the bran/shorts had the highest phenolic content followed by the whole seed, germ, and endosperm. Lutein was the predominant carotenoid found in whole wheat and its milled fractions. Zeaxanthin, β-carotene, and β-cryptoxanthin were also detected. Mainly α- and β-tocopherols and tocotrienols were found in all varieties of whole wheat. The bran/shorts fraction consisted mainly of tocotrienols, though the germ fraction

consisted mainly of tocopherols. The bran/shorts had the highest insoluble-bound ORAC followed by the whole seed, germ, and endosperm. Free phenolics extracts from the germ and insoluble-bound phenolics extracts from whole wheat and its milled fractions significantly inhibited the proliferation of HepG2 cells after 96 hours at 10 mg/mL.

The data provided in this dissertation, supports the idea that the potential health benefit in the lower gastrointestinal tract from whole wheat consumption is due to the antioxidant activity of phenolics compounds from the insoluble-bound fraction of whole wheat. This dissertation also lays the foundation for future research regarding the potential health benefit of whole wheat phytochemicals.

BIOGRAPHICAL SKETCH

Neal Okarter received a Bachelor of Science degree in Nutritional Sciences from Cornell University. At Cornell, he was an undergraduate teaching assistant for two classes and a member of the Men's Track and Field team. Neal also received a Master of Science degree in Biological Chemistry from the University of Exeter. At Exeter, Neal conducted research regarding the pro and antioxidant activity of chalcogen-containing compounds and co-authored two research articles. After returning to Cornell for further graduate study, Neal was awarded the Lydia Leuder Darling Scholarship, the Charles Quillman Fellowship, the SUNY Underrepresented Minority Graduate Fellowship, the Provost's Diversity Fellowship, and the Ruth and Henry Herzog Graduate Award. Neal also served as the Graduate Student Representative on the College of Agricultural and life Sciences Diversity Committee.

This dissertation is dedicated to my family.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

LIST OF FIGURES

sterile water in growth medium. All controls and treatments were prepared in the absence of phenolic red. After 24 or 96 hours, the medium was collected. LDH activity was determined as described in the Materials and Methods section of this paper. * Indicates a significant difference from the medium control at the respective time point ($p < 0.05$).

LIST OF TABLES

LIST OF ABBREVIATIONS

ABAP	2,2'-azobis (2-amidinopropane) dihydrochloride
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
BMI	Body mass index
CAA	Cellular antioxidant activity
CHD	Coronary heart disease
CI	Confidence interval
CVD	Cardovascular disease
DAD	Diode array detector
DCFH-DA	Dichlorofluorescein diacetate
DMEM	Delbucco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DW	Dry weight
ESI-MSMS	Electrospray ionization – mass spectrometry mass spectrometry
FBS	Fetal bovine serum
FD	Fluorescence detector
FDA	Food and Drug Administration
FRAP	Ferric ion reducing antioxidant power
FW	Fresh weight
GAE	Gallic acid equivalents
HDL	High density lipoprotein
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

HPLC	High performance liquid chromatography
IHD	Ischemic heart disease
IMT	Intimal medial thickness
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
M	Molar (moles per liter)
np	Normal phase
ORAC	Oxygen radical absorbance capacity
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PSC	Peroxyl radical scavenging capacity
rp	Reversed phase
RR	Relative risk
SD	Standard deviation
SDS	Sodium dodecyl sulfate
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant capacity
THF	Tetrahydrofuran
TOSC	Total oxyradical scavenging capacity
TPC	Total phenolic content
TRAP	Total peroxyl radical-trapping antioxidant parameter
UV	Ultra violet

LIST OF SYMBOLS

Σ The sum of all

PREFACE

A whole grain consists of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components - the starchy endosperm, germ and bran - are present in the same relative proportions as they exist in the intact caryopsis. Epidemiological studies have consistently shown that consumption of whole grain is associated with reduced risk of cardiovascular disease, type II diabetes, and some cancers.

The 2005 Dietary Guidelines for Americans recommends Americans to consume at least three ounce-equivalents of whole grain products each day, being sure to "make half your grains whole". However, Americans are only eating 34% of the recommended whole grain consumption, compared to 177% of the recommended refined grain consumption. As a result, Americans may be losing the potential health benefit of whole grain consumption. Therefore, there is a need to add functional ingredients to refined grains in order to restore the potential health benefit of whole grain consumption.

CHAPTER 1

HEALTH BENEFITS OF WHOLE GRAIN PHYTOCHEMICALS[1]

Neal Okarter and Rui Hai Liu

Abstract

A whole grain consists of the intact, ground, cracked or flaked caryopsis,
whose principal anatomical components - the starchy endosperm, germ and bran - are
present in the same relative proportions as they exist in the intact caryopsis. Whole
grain food products can be intact, consisting of the original composition of bran, germ,
and endosperm, throughout the entire lifetime of the product, or reconstituted, in
which one or more of the original components of a whole grain is recombined to the
relative proportion naturally occurring in the grain kernel.   Increased consumption of
whole grains has been associated with reduced risk of major chronic diseases
including cardiovascular disease, type II diabetes, and some cancers. Whole grain
foods offer a wide range of phytochemicals with health benefits that are only recently
becoming recognized. The unique phytochemicals in whole grains are proposed to be
responsible for the health benefits of whole grain consumption. In this paper, whole
grain phytochemicals and the health benefit associated with their consumption are
reviewed.

1.1 Introduction

Epidemiological studies have consistently shown that consumption of whole grain is associated with reduced risk of cardiovascular disease (CVD) (*1-3*), type II diabetes (*4-6*), and some cancers (*7, 8*). Because of the increased evidence indicating the health benefits of whole grain, the recommendation for whole grain consumption has been changed. In 1995, the USDA's Dietary Guidelines for Americans advised Americans to choose a diet with plenty of grain products, recommending 6 to 11 servings of grain products and several servings of whole grain breads and cereals (*9*). Although this recommendation did recognize the significance of whole grain consumption, emphasis and specificity were not placed on whole grain consumption. The 2005 Dietary Guidelines for Americans recommends Americans to consume at least three ounce-equivalents of whole grain products each day, being sure to "make half your grains whole" (*10*). The recognition of the potential health benefits of whole grain consumption is a step in the right direction for the improved health of the American public.

Industry has responded to the increased recommendation for whole grain consumption. Although consumption of all-wheat flour dropped in 2006, there were 446 new whole grain food products introduced into the market between 2003 and 2004 (*11, 12*). Nearly ten times more whole grain food products were launched in 2006 than in 2000 (*13*).

The objectives of this paper are to review the current literature on whole grains, especially on studies linking whole grain consumption and health benefits, and whole grain phytochemicals.

1.2 Definitions of Whole Grains and Whole Grain Products

A whole grain consists of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components - the starchy endosperm, germ and bran - are present in the same relative proportions as they exist in the intact caryopsis (*14*). The endosperm contains the food supply for the germ and provides energy for the rest of the plant. The endosperm is the largest component and contains starchy carbohydrates, proteins, vitamins and minerals. The bran is the multi-layered outer skin of the grain that protects the germ and the endosperm from damage due to sunlight, pests, water, and diseases. It contains phenolic compounds, vitamins, minerals, and fiber. The germ refers to the embryo, the part of the grain that becomes a new plant when fertilized by pollen. It contains vitamins, some protein, minerals, and fats.

Whole grain products can be defined by one of two definitions. An intact whole grain product is a product that has the original composition of bran, germ, and endosperm throughout the entire lifetime of the product, from field to consumption. A reconstituted whole grain product is a product that has the original components of a whole grain recombined to the relative proportion naturally occurring in the grain kernel. Due to advances in food processing and the commonplace nature in which these processes take place, the bulk of the whole grain food products found on shelves would be considered reconstituted whole grain products.

There are many grains such as wheat, corn, barley, and rice, both whole and refined, which are consumed on a daily basis in a number of products from around the world. Wheat has become the prominent grain based on consumption. There are two types of wheat that are common in foods typically eaten in North America and Europe, durum wheat, *Triticum turgidum durum*, which is used in pasta products and bread,

and *Triticum aestivum vulgare*, which is used in most other wheat products. Wheat

varieties are characterized by the hardness and color of the bran and the season in

which they are planted. Hard red winter wheat cultivars are most popular due to high

gluten content, making them particularly good for cookies and some bread (*15*). White

wheat cultivars, both hard and soft, are often exported from North America for use in

making flat breads and noodles (*16*). Gluten content has been used as basis for

determining wheat quality (*15*). Spelt, *Triticum aestivum spelta*, is a variety of wheat

that typically has higher protein content than wheat. Corn, *Zea mays L.*, is another

commonly eaten grain that recently has gained more attention because of its

antioxidant content. Rice, *Oryza sativa L.*, is the major staple for a majority of the

world's population. Rice is rarely eaten as a whole grain. Generally, the endosperm

fraction, polished rice, without the bran and germ fractions, is eaten. Rice can also be

parboiled, incorporating B vitamins into the endosperm of the grain. White rice is not

considered a whole grain. Oats, *Avena sativa L.*, are almost always eaten whole since

their bran and germ fractions are rarely removed. They also tend to have a sweet taste,

making good breakfast cereals and beers. Millet, *Panicum miliaceum L.*, is rarely

consumed by humans in North America, but is very common in Asia. In the United

States, it is mainly used as bird feed or mixed with other grains. Barley, *Hordom

vulgare L.*, has a tough hull that is difficult to remove and therefore has long cooking

times. Rye, *Secale cereale L.*, has high fiber content in its endosperm and is

consumed with highest frequency in parts of Scandinavia (**Table 1.1**).

   1.3 Health Benefits of Whole Grains

   Whole grain consumption has been associated with reduced risk of CVD, type

II diabetes, obesity and some cancers.

Table 1.1 – Common whole grains and food products

Species	Common Name	Common Food Products
Triticum aestivum	Wheat	Breads, flours, pasta, baked goods
Zea mays	Corn	Corn cakes, tortilla, popcorn, hominy
Orize sativa	Rice	White rice, brown rice, parboiled rice
Avena sativa	Oats	Oatmeal, flour
Panicum miliaceum	Millet	Bird food, porridge, millet
Hordom vulgare	Barley	Hulled barley
Triticum aestivum spelta	Spelt	Breads, baked goods
Secale cereale	Rye	Breads

1.3.1 Whole Grain Consumption and Risk of CVD

Epidemiological studies have consistently shown that consumption of whole grains is associated with reduced risk of CVD (**Figure 1.1**). Pietinen *et al* reported results from the Alpha-Tocopherol, Beta-Carotene Lung Cancer Prevention Study and showed that total dietary fiber was inversely associated with risk of coronary death. Further, a ten-gram per day higher intake of fiber appeared to lower the risk of coronary death by 17% (RR = 0.83; 95% CI = 0.80 – 0.86) (*17*). Rye products, which make up a large part of the whole grain intake in the study population, were also inversely associated with coronary death.

Jacobs *et al* reported results from the Iowa Women's Health Study and showed that when the highest category of whole grain intake was compared to the lowest there was an inverse association between whole grain intake and risk of death from ischemic heart disease (IHD), even after adjustment for potentially confounding factors and adjustment for dietary fiber intake (RR = 0.70) (*2*). This association was independent of intake of refined grains. Specifically, these finding held true for dark bread and whole grain breakfast cereals, but not for wheat germ, cooked oatmeal, bulgar, kasha, and couscous. After adjustment for total dietary fiber intake, Jacobs *et al* showed there was an inverse association between whole grain intake and IHD across all intakes of whole grain, RR = 0.77; 95% CI 0.54 – 1.10 for highest quintile of whole grain intake (*2*).

Liu *et al* reported results from the Nurse's Health Study (NHS) and showed that there was a strong inverse association between whole grain intake and risk of coronary heart disease (CHD) (RR = 0.51; 95% CI 0.41 – 0.64), for women in the highest quintile for whole grain consumption when compared to the lowest (*3*). This association was observed for both nonfatal CHD and fatal CHD (*3*). Higher intake of refined grains was also associated with greater risk of CHD in women who had never

smoked (RR = 1.46), although this association was attenuated after adjustment for CHD risk factors and the inclusion of whole grain intake into the model. In age-adjusted analyses, Wolk *et al* showed women in the highest quintile of long term dietary fiber intake had 43% lower risk of nonfatal myocardial infarct and 59% lower risk of fatal coronary disease in comparison to those in the lowest quintile. Further, each ten-gram increase in long-term fiber consumption correlated to the reduced risk of total CHD (RR = 0.81; 95% CI 0.66 – 0.99) (*18*). Wolk *et al* also showed that women in the highest category of long term dietary fiber intake as compared to the lowest category had a 34% lower risk of total CHD. With regard to specific food products, consumption of cold breakfast cereal five or more times per week in comparison to no cold breakfast cereal was associated with a 19% lower risk of total CHD (RR = 0.81; 95% CI 0.62 – 1.06). This association was even stronger for oatmeal, having a 29% lower risk of total CHD (RR = 0.71; 95% CI 0.38 – 1.34) (*18*).

The Adventist Health Study investigated incidences of chronic disease in relation to lifestyle in Seventh Day Adventists, a population that has a lower risk of fatal CHD when compared with others from California possibly due to adherence to a lacto-ovo vegetarian diet and lower rates of smoking (*19*). Fraser *et al* reported that although the food that was most consistently associated with reduced risk of IHD was nuts, preference for whole-grain bread instead of preference for white bread was also associated with reduced risk of IHD (*1*). After adjustment for non-dietary risk factors and consumption of other foods, those who preferred whole-grain bread had a relative risk of 0.89 for fatal IHD and 0.56 for nonfatal IHD when compared to those who had a preference for white bread.

Anderson *et al* performed a meta-analysis of studies that investigated the relationship between whole grain consumption and the risk of CHD. Of the twelve studies analyzed, four studies (when the data was pooled) showed that there was a

significant inverse association between whole grain and whole wheat bread consumption and reduced risk of CHD (RR = 0.74; 95% CI 0.64 – 0.84), after adjustment for primary and secondary risk factors (*20*).

Pereira *et al* performed a meta-analysis of studies that investigated the relationship between dietary fiber and risk of CHD. After adjustment, Pereira showed a significant inverse association between dietary fiber and risk of CHD (RR = 86; 95% CI 0.78 – 0.96) (*21*).

Erkkilä *et al* investigated the association between cereal fiber and whole grain consumption and progression of coronary artery atherosclerosis in postmenopausal women with coronary artery disease using the data from the Estrogen Replacement and Atherosclerosis trial. The minimum coronary artery diameter (MCAD) was used as a measure of progression of coronary artery atherosclerosis. Erkkilä *et al* reported that MCAD decreased less in those who consumed more than the median intake of cereal fiber compared to those who at less than the median intake of cereal fiber, after adjusting for cardiovascular disease, blood pressure, and other possible confounding factors (*22*). This study shows that whole grain consumption has health benefit not only for those who don't have cardiovascular disease but also for those that do have cardiovascular disease.

Lockheart *et al* investigated the association between dietary patterns and risk of first myocardial infarction using the data from a case-control study performed in Norway. After adjusting for family history of heart disease, smoking, energy intake, and other possible confounding factors, whole grain breakfast cereals were inversely associated with risk of first myocardial infarction (RR = 0.64; 95% CI = 0.45 – 0.90) (*23*) when comparing the group with the highest level of whole grain intake to the group with the lowest.   However, whole grain breads were not inversely associated with risk of first myocardial infarction (RR = 0.94; 95% CI = 0.67 – 1.32). This is

most likely due to the fact that whole grain bread (heavy wholegrain bread, dark flat bread, and medium wheat bread) consumption is common in Norway.

Wang *et al* investigated the association between whole grain consumption and hypertension using data from the US Health Professional's Follow-Up Study. After adjusting for possible confounding lifestyle, clinical, and dietary factors, whole grain consumption was inversely associated with hypertension when comparing the highest quintile of whole grain intake to the lowest (RR = 0.89; 95% CI = 0.82 – 0.97) (*24*). No significant association was seen between refined grain consumption and hypertension.

Lutsey *et al* investigated the association between whole grain consumption and common carotid artery intimal medial thickness (IMT) as a measure of subclinical cardiovascular disease using data from Multi-Ethnic Study of Atherosclerosis. After adjusting for fruit, vegetable, and refined grain intakes and other possible confounding factors, whole grain consumption was not associated with common carotid artery IMT, when comparing the highest quintile of whole grain consumption to the lowest (*25*). Associations between whole grain consumption and common carotid artery IMT were not found possibly because subclinical cardiovascular disease markers are weakly correlated with chronic diseases. Further, the cross-sectional design of the study may have made finding the association between whole grain consumption and common carotid artery IMT difficult.

Flint *et al* investigated the association between whole grain consumption and hypertension in men using data from the Health Professionals Follow-Up Study. After adjusting for fruit and vegetable consumption, smoking, family history of hypertension, physical activity and other possible confounding factors, whole grain consumption was associated with reduced incident of hypertension, when comparing

the highest quintile of whole grain consumption to the lowest (RR = 0.81, 95% CI = 0.75-0.87) (*26*).

Nettleton *et al* used the Multi-Ethnic Study of Atherosclerosis (MESA) to investigate the association between whole grain and fruit consumption and relative risk of cardiovascular disease. After adjusting for energy intake, sex, age, race and ethnicity, and education, whole grain and fruit consumption was inversely associated with reduced risk of cardiovascular disease (RR = 0.37; 95% CI = 0.19-0.72) (*27*). Further, there was an inverse association between whole grain and fruit consumption and reduced risk of myocardial infarction (RR = 0.34; 95% CI = 0.12-0.94) (*27*). However, there was association between whole grain and fruit consumption and coronary heart disease.

1.3.2 Whole Grain Consumption and Risk of Type II Diabetes and Obesity

Lifestyle modification and weight control are major factors in the prevention and treatment of diabetes. Several large epidemiological studies have linked whole grain consumption with reduced risk of type II diabetes (**Figure 1.2**). Salmerón *et al* investigated the relationship between dietary fiber and risk of type II diabetes. Only cereal fiber was inversely associated with risk of type II diabetes (RR = 0.70, 95% CI = 0.51 – 0.96) when comparing the highest quintile of cereal fiber intake to the lowest (*6*). Total dietary fiber consumption was also inversely associated with risk of type II diabetes, but the association was not significant.

Liu *et al* investigated the association between whole and refined grain intake and risk of type II diabetes. After adjusting for age and energy intake, it was found that there was a significant inverse association between whole grain intake and risk of type II diabetes, when comparing the highest quintile of whole grain intake to the lowest quintile (RR = 0.62, 95% CI = 0.53 – 0.71) (*4*). There was a positive
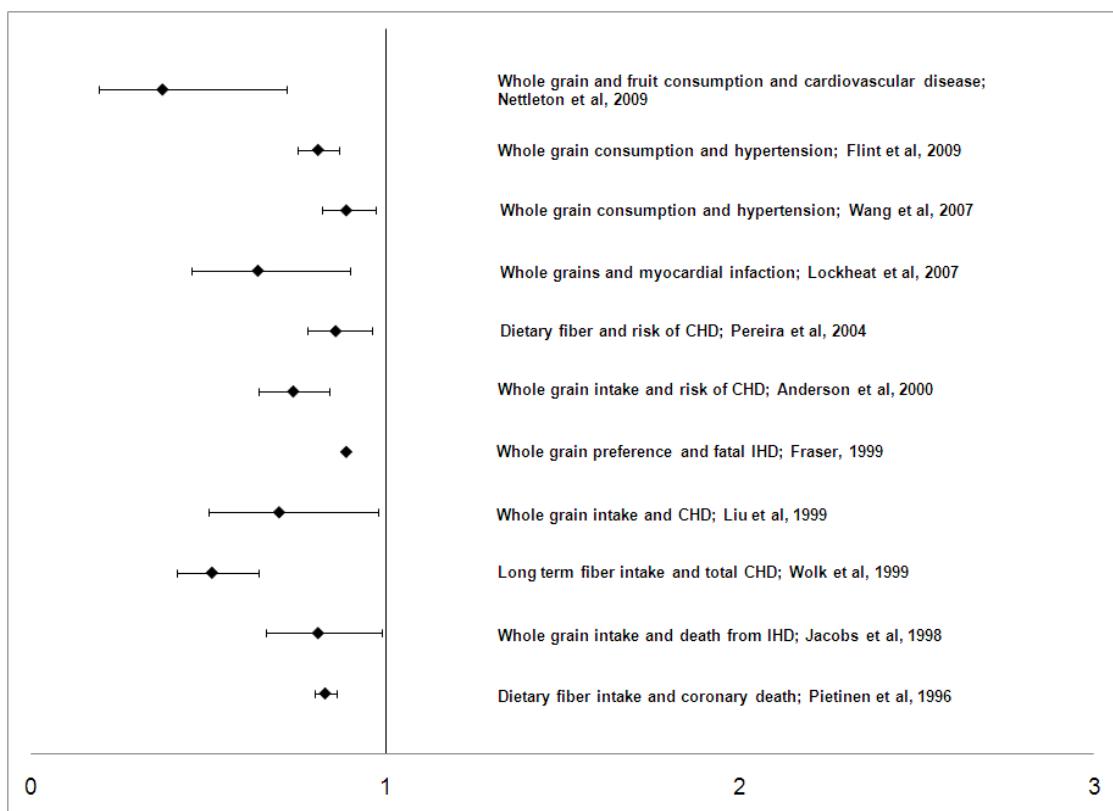
Figure 1.1 – Consumption of whole grains or dietary fiber and relative risk of cardiovascular disease. Relative risk ± 95% confidence interval.

association between refined grain intake and risk of type II diabetes, when the highest quintile of refined grain intake was compared to the lowest quintile after adjusting for age and energy intake (RR = 1.31, 95% CI = 1.12 – 1.53) (*4*). Those in the highest quintile of refined grain to whole grain intake had a significantly higher risk of type II diabetes when compared to those in the lowest quintile of refined grain to whole grain intake (RR = 1.57, 95% CI = 1.36 – 1.82) (*4*). This provided further supportive evidence that consumption of whole grains is associated with reduced risk of developing type II diabetes.

Meyer *et al* investigated the association between cereal fiber and whole grain consumptions and relative risk of type II diabetes. Whole grain consumption was inversely associated with risk of type II diabetes when comparing the highest quintile of whole grain intake to the lowest (RR = 0.79; 95% CI = 0.65 – 0.96). Cereal fiber consumption was also inversely associated with risk of type II diabetes when comparing the highest quintile of cereal fiber consumption to the lowest (RR = 0.64; 95% CI = 0.53 – 0.79). Refined grain intake was positively associated with risk of type II diabetes when comparing the highest quintile of refined grain intake to the lowest but the association was not as strong as that of whole grains or cereal fiber (RR = 0.87; 95% CI = 0.70 – 1.08; P for trend = 0.36) (*5*).

van Dam *et al* examined the association between dietary patterns and risk for type II diabetes, using data from the Health Professionals Follow-Up Study. The sample population was divided into one of two dietary patterns (prudent or western patterns) based on the composition of the diet. The prudent dietary pattern was characterized by high consumption of whole grains, fruits, vegetables, seafood, legumes, white meat, nuts and plant oils. The western dietary pattern was characterized by high consumption of refined grains, red meat, processed meat, potatoes, and foods with high saturated fats, cholesterol, and trans fats. After

modeling western pattern foods simultaneously, refined grain intake remained positively associated with risk of type II diabetes (RR = 1.32, 95% CI = 1.09 – 1.60) (*28*).

Fung *et al* investigated the association between whole grain consumption and risk of type II diabetes, using the data from the Health Professionals Follow-Up Study. After adjusting for fruit and vegetable consumption and other confounding factors, whole grain consumption was inversely associated with risk of type II diabetes when the highest quintile of whole grain intake was compared to the lowest (RR = 0.70; 95% CI = 0.57 – 0.85). Refined grain intake was positively associated with risk of type II diabetes when comparing the highest quintile of refined grain intake to the lowest (RR = 1.08; 95% CI = 0.87 – 1.33) (*29*).

Liu *et al* also used data from the Nurses Health Study to investigate the association between whole grain intake, body weight, and long-term body weight changes. They found that women in the highest quintile of dietary fiber intake had a 49% reduced risk of major weight gain when compared to women in the lowest quintile of dietary fiber (RR = 0.51, 95% CI = 0.39 – 0.67) (*30*). Further, women who consumed larger amounts of whole grain consistently weighed less than women who consumed smaller amounts of whole grain.

Montonen *et al* investigated the association between whole grain intake and risk of type II diabetes using data from the Finnish Mobile Clinic Health Examination Survey. After adjusting for fruit, berry, and vegetable consumption and other confounding factors, whole grain consumption was inversely associated with type II diabetes when compared the highest quartile of whole grain consumption to the lowest (RR = 0.65; 95% CI = 0.36 – 1.18). Cereal fiber consumption was inversely associated with risk of type II diabetes when the highest quartile of cereal fiber intake was compared to the lowest (RR = 0.39; 95% CI = 0.20 – 0.77) (*31*).

Schulze *et al* investigated the association between cereal fiber intake and incidence of type II diabetes using data from the Nurse's Health Study. After adjusting for familial history of type II diabetes, body mass index, and other confounding factors, cereal fiber consumption was inversely associated with incidence of type II diabetes when comparing the highest quintile of cereal fiber intake to the lowest (RR = 0.64; 95% CI = 0.48 – 0.86) (*32*).

Bazzano *et al* investigated the association between whole and refined grain breakfast cereal consumption and weight gain in men using data from the Physician's Health Study. After adjusting for baseline BMI, physical activity, age, and other possible confounding factors, whole grain breakfast cereal consumption was inversely associated with risk of having a BMI greater than 25 (RR = 0.83; 95% CI = 0.71 – 0.98) and body weight gain of more than 10 kg (RR = 0.78; 95% CI = 0.64 – 0.96), 8 years after initial subject evaluation (*33*). This inverse association was independent of the type of grains that constituted the whole grain breakfast cereal. The data suggest that whole grain breakfast cereals are an important part of the prudent dietary pattern.

Munter *et al* investigated the association between whole grain, bran, and germ intake using data from the first and second trials of the Nurse's Health Study. After adjusting for physical activity, total energy intake, and other possible confounding factors, whole grain intake was inversely associated with risk of type II diabetes in the first trial of the Nurse's Health Study (RR = 0.63; 95% CI = 0.57 – 0.69) and in the second trial of the Nurse's Health Study (RR = 0.68; 95% CI = 0.57 – 0.86) (*34*). Similar associations were found between bran consumption and risk of type II diabetes in both trials Nurse's Health study after adjusting for physical activity, total energy intake, and other possible confounding factors (RR = 0.57; 95% CI = 0.51 – 0.63 and RR = 0.64; 95% CI = 0.54 – 0.76, respectively) (*34*).

Newby *et al* investigated the association between whole grain consumption and BMI, weight, and waist circumference using data from the Baltimore Longitudinal Study on Aging. Whole grain and cereal fiber intake were inversely associated with BMI, weight, and waist circumference when the highest quintile of whole grain or cereal fiber intake was compared to the lowest after adjusting for refined grain intake, total energy intake, percentage energy from saturated fat, and other possible confounding factors (*35*).

Nettleton *et al* used the Multi-Ethnic Study of Atherosclerosis (MESA) to investigate the association between whole grain and fruit consumption and relative risk of type II diabetes. After adjusting for energy intake, sex, age, race and ethnicity, and education, whole grain and fruit consumption was inversely associated with reduced risk of type II diabetes (RR = 0.63; 95% CI = 0.45-0.89) (*36*).

1.3.3 Whole Grain Consumption and Risk of Cancers

Many large epidemiological studies have investigated the association between whole grain consumption and relative risk of cancers (**Figure 1.3**). Previous studies have shown an association between increased consumption of whole grains and colorectal and breast cancers (*7, 37*). A case control study was performed in southern Australia to investigate the association between diet and breast cancer. After adjusting for energy intake and other risk factors, Rohan *et al* found that there was little association between dietary fiber and the risk of death from breast cancer as determined by hazard ratio across various levels of intake. Consumption of between 23 and 26 g dietary fiber led to a relative risk of 1.12 (95% CI 0.60 – 2.07), while consumption greater than 27 g of dietary fiber led to a relative risk of 0.87 (95% CI 0.45 – 1.68), p = 0.812 for trend (*37*). The data suggest that there is an unclear association between dietary fiber and risk of death from breast cancer.
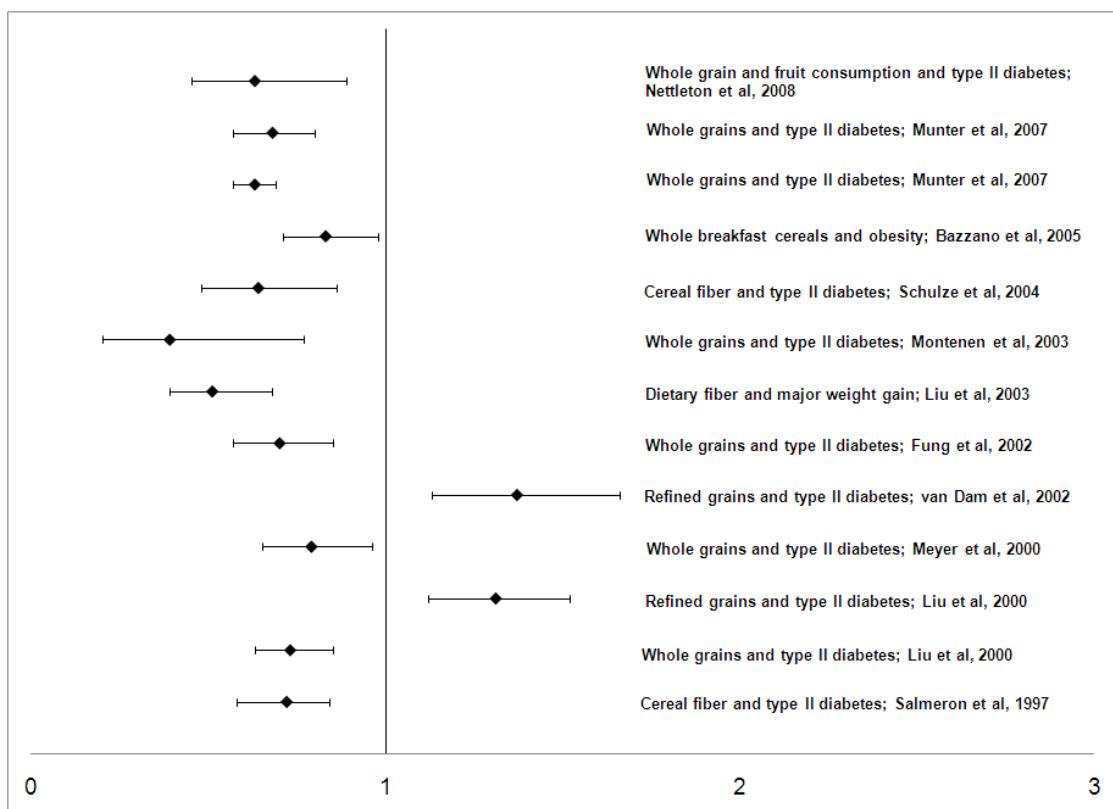
Figure 1.2 – Consumption of whole grain or dietary fiber and relative risk of type II diabetes, obesity, or weight gain. Relative risk ± 95% confidence interval.

Nicodemus *et al* investigated the association between increased consumption of dietary fiber and risk of breast cancer. Women in the highest quintile of whole grain intake had a 23% higher risk of breast cancer incidence than women in the lowest quintile of whole grain intake. Further, there was no association between whole grain intake and risk of breast cancer incidence in women who had not received a mammography before 1989 (*38*). This finding was similar to that of a study reported by Willett *et al* (*39*), which found a slightly increased, but non-significant risk of breast cancer in postmenopausal women who consumed more dietary fiber.

Pietinen *et al* investigated the association between consumption of dietary fiber and risk of colorectal cancer using data from the Alpha-Tocopherol, Beta-Carotene Lung Cancer Prevention Study and found that there was no association between colorectal cancer and intake of total dietary fiber, soluble fiber, or insoluble fiber from the various rye products (*40*). Further, there was no association between vegetable, cereal, or fruit intake and risk of colorectal cancer. These findings are contrary to the general convention that increased consumption of fruits, vegetables, and whole grains decrease the risk of cancer, specifically colorectal cancer.

Kasum *et al* investigated the association between whole grain consumption and risk of endometrial cancer, using data from the Iowa Women's Health Study. After adjusting for refined grain, fruit, vegetable, and red meat consumption and other confounding factors, there was no association between whole grain consumption and risk of endometrial cancer (RR = 0.99; 95% CI = 0.71 – 1.38). However, for women who had never had any hormone replacement therapy, whole grain consumption was inversely associated with risk of endometrial cancer when the highest quintile of whole grain consumption was compared to the lowest (RR = 0.63; 95% CI = 0.39 – 1.01) (*41*).

Some studies have shown that there is an inverse association between whole grain consumption and risk of colorectal cancer (*7, 42*). Larsson *et al* investigated the association between whole grain consumption and colon cancer using data from the Swedish Mammography Cohort. After adjusting for red meat, fruit, and vegetable consumption and other possible confounding factors, whole grain consumption was inversely associated with risk of colon cancer (RR = 0.67; 95% CI = 0.47 – 0.96) when comparing the highest quintile of whole grain intake to the lowest (*43*).

Schatzkin *et al* showed there was an inverse association between whole grain consumption and risk of colorectal cancer (RR = 0.79; 95% CI = 0.70 – 0.89) after multivariate analysis (*44*). This association was stronger for men (RR = 0.79; 95% CI = 0.68 – 0.91) than for women (RR = 0.87; 95% CI = 0.70 – 1.07) (*44*). The association between whole grain consumption and reduced risk of site specific tumors was strongest for the rectum (RR = 0.64; 95% CI = 0.51 – 0.81) (*44*).

Chan *et al* investigated the association between whole grain consumption and risk of pancreatic cancer using data from a large population-based case-control study on pancreatic cancer. After adjusting for history of smoking, red meat, fruit, and vegetable consumption, and other possible confounding factors, whole grain consumption was inversely associated with risk of pancreatic cancer (RR = 0.60; 05% CI = 0.30 – 1.2), when comparing the highest quartile of whole grain intake to the lowest (*45*).

Sonestedt *et al* investigated the association between cereal consumption and risk of invasive breast cancer using data from Malmö Diet and Cancer Cohort Study. After adjusting for age, smoking, total energy, and other possible confounding factors, cereal consumption and high-fiber bread consumption were not associated with reduced risk of invasive breast cancer (*46*).

Williams *et al* investigated the association between whole grain consumption and risk of rectal cancer in both Whites and African-Americans using data from North Carolina Cancer Study – Phase II. After adjusting for age, non-steroidal anti-inflammatory drug use, total energy, and other possible confounding factors, whole grain consumption was not significantly associated with reduced risk of rectal cancer in White-Americans (RR = 0.93; 95% CI = 0.66-1.31) or African-Americans (RR = 0.67; 95% CI = 0.21-1.42) (*47*).

1.4 Whole Grain Phytochemicals

Phytochemicals are defined as bioactive non-nutrient plant compounds in fruits, vegetables, whole grains, and other plant foods that have been associated with reduced risk of major chronic diseases (*48*). Whole grains contain many phytochemicals with health benefits that are only recently becoming recognized. The most important groups of whole grain phytochemicals are phenolics (phenolic acids, alkylresorcinols, and flavonoids), carotenoids, vitamin E, γ-oryzanols, dietary fiber, and β-glucan.

1.4.1 Phenolics

The most studied whole grain phytochemicals are phenolics. Phenolics are compounds with one or more aromatic rings and one or more hydroxyl groups. Included in this group of compounds are phenolic acids, alkylresorcinols, and flavonoids.
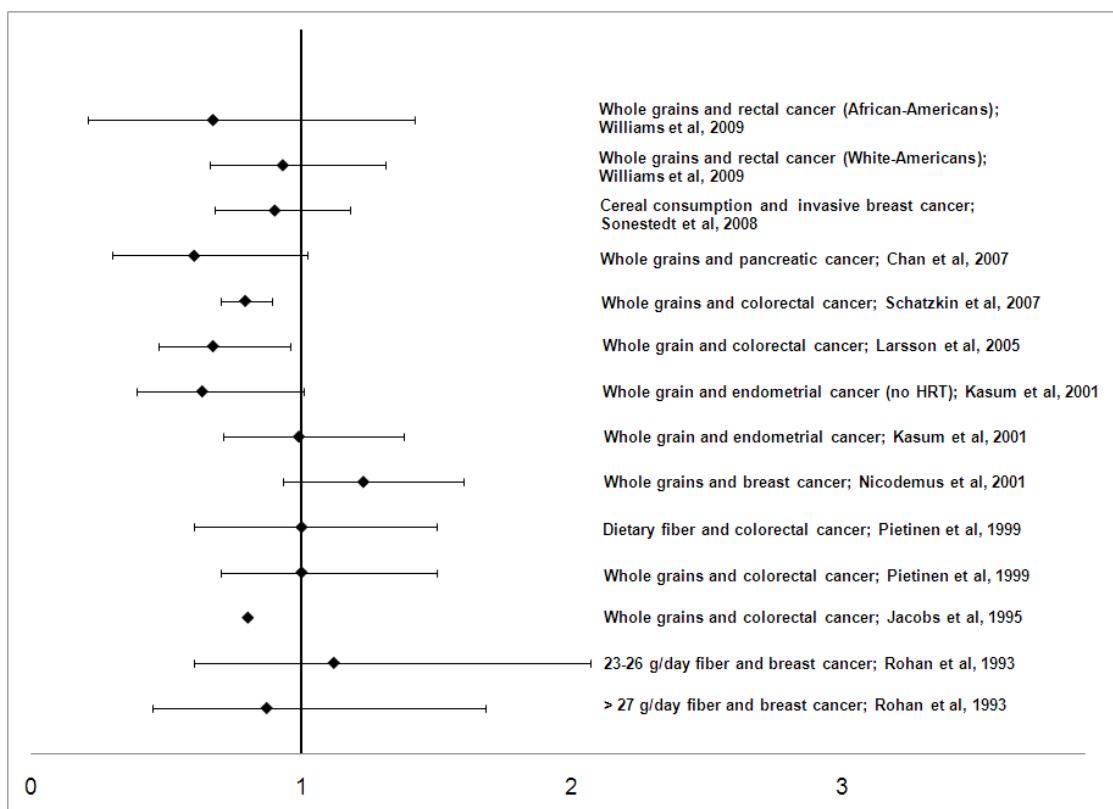
Figure 1.3 – Consumption of whole grain or dietary fiber and relative risk of cancers. Relative risk ± 95% confidence interval.

1.4.1.1 Total Phenolics

Zieliński and Kozlowska (*49*) reported the total phenolic content in seven cereal grains and their various fractions. The total phenolic content of the whole grains studied ranged from 1.5 μg catechin equivalents/mg of lyophilizate in oat (Slwako) to 11.3 μg catechin/mg of lyophilizate in barley (Mobek). When using a different extraction procedure, the total phenolic content of the whole grains studied ranged from 8.9 μg catechin equivalents/mg of lyophilizate in rye (Dañkowski Zlote) to 117.7 μg catechin equivalents/mg of lyophilizate in buckwheat (Kora) (*49*).

The previous study and others greatly underestimated the total phenolic content of the grain samples by using finely powdered samples and long extraction times in an attempt to maximize the extraction of phenolic compounds from the grains. However, these studies only extracted soluble phenolic compounds and excluded insoluble-bound phenolic compounds, compounds that are esterified to macromolecules and capable of surviving digestion in the upper gastrointestinal tract (*50*). As Adom and Liu mentioned, as high as 74% of phenolic compounds of a whole grain can be found in the insoluble bound fraction, as is the case for wheat, corn, oat, and rice (*50*).

Sosulski *et al* investigated the phenolic acid content, including free phenolic acids, soluble-conjugated phenolic acids and bound phenolic acids in rice, oats, wheat, and corn flours (*51*). Although this study did recognize the existence of phenolic acids in all three forms (free, soluble-conjugated, and insoluble), the study did not investigate the total phenolic content of all three forms.

Adom and Liu determined the phytochemical profiles in all three forms of four grains, corn, wheat, oats, and rice. Corn had the most total phenolic content at 1560 ± 60 μmol gallic acid equivalents/100 g, followed by wheat at 800 ± 40 μmol gallic acid equivalents/100 g, oats at 650 ± 20 μmol gallic acid equivalents/100 g and lastly rice

at 560 ± 20 µmol gallic acid equivalents/100 g (*50*). Corn had the most total phenolic

content in the bound fraction at 1340 ± 60 µmol gallic acid equivalents/100 g,

followed by wheat at 610 ± 40 µmol gallic acid equivalents/100 g, oats at 480 ± 10

µmol gallic acid equivalents/100 g and rice at 350 ± 10 µmol gallic acid

equivalents/100 g (**Figure 1.4**) (*50*). Bound phenolics contributed 85% of the total

phenolic content in corn, 76% in wheat, 75% in oats, and 62% in rice (*50*).

Adom *et al* (*52*) determined the phytochemical distribution in the milled

fractions (endosperm and bran/germ) of different wheat varieties and showed that the

majority of health beneficial phytochemicals of whole wheat grains were present in

the bran/germ fraction. The total phenolic content in the bran/germ ranged from 2870

to 3120 µmol gallic acid eq./100 g compared to 180 to 200 µmol gallic acid eq./100 g

in the endosperm (*52*). The data show that in whole grain wheat flour, the bran/germ

fraction contributed 83% of total phenolic content.

1.4.1.2 Phenolic Acids

Phenolic acids are hydroxylated compounds that are derived from benzoic acid

or cinnamic acid, with derivatives of the latter being more common (**Figure 1.5**).

These compounds have been partially attributed to the positive physiological effects of

whole grains consumption because of their unique composition and antioxidant

activities. Phenolic acids, which are found mainly in the outer layer of grains, can be

found as part of a complex structure such as lignin (see below) or as a sugar

derivative.

Ferulic acid, a derivative of cinnamic acid, is the most abundant phenolic acid

in grains. Adom and Liu reported the ferulic acid content of corn, wheat, oats and rice

(*50*). Corn had the highest total ferulic acid content at 906 ± 9 µmol ferulic acid/100

g, followed by wheat at 333 ± 16 µmol ferulic acid/100 g, oats at 185 ± 5 µmol ferulic
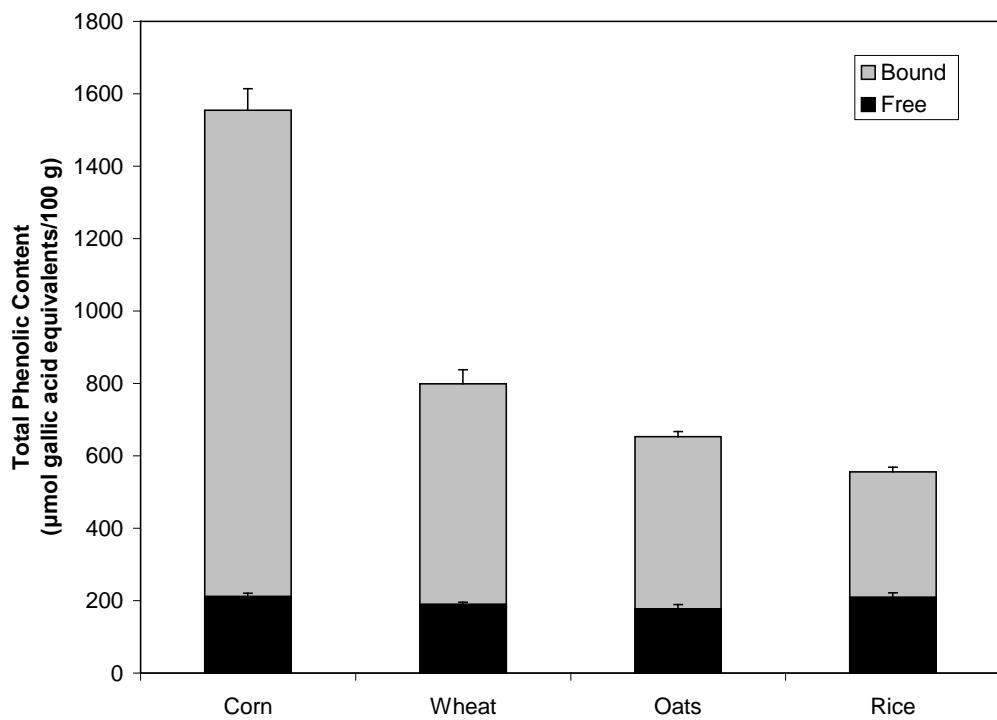
Figure 1.4 – Total phenolic content of whole grains. Adapted from Adom and Liu (*50*).

Hydroxybenzoic Acids
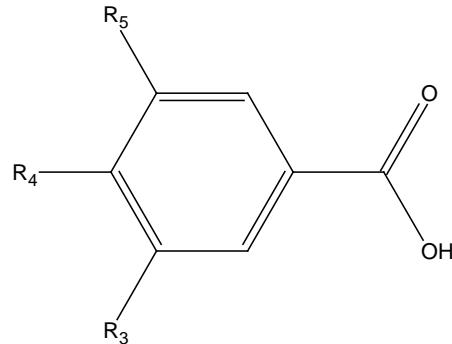
	R$_3$	R$_4$	R$_5$
p-Hydroxybenzoic Acid	H	OH	H
Gallic Acid	OH	OH	OH
Syringic Acid	OCH$_3$	OH	OCH$_3$

Hydroxycinnamic Acids
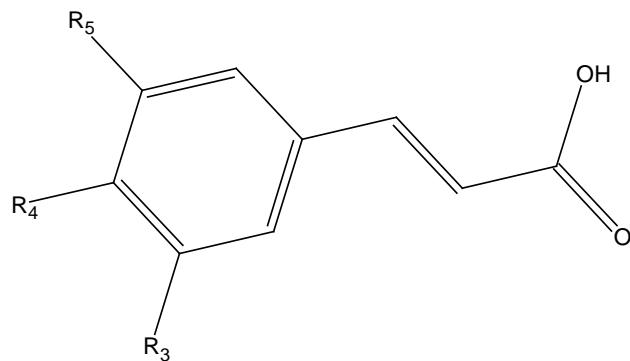
	R$_3$	R$_4$	R$_5$
p-Coumaric Acid	H	OH	H
Ferulic Acid	OCH$_3$	OH	H
Sinnapic Acid	OCH$_3$	OH	OCH$_3$

Figure 1.5 – Structure of phenolic acids: benzoic acid and cinnamic acids derivatives.

acid/100 g, and rice at 154 ± 9 μmol ferulic acid/100 g (*50*). More than 93% of the total ferulic acid content was found in the insoluble-bound fraction. Adom and Liu found that the ratio of free:soluble conjugated:insoluble-bound ferulic acid was 0.1:1:100 (*50*).

Other phenolic acids have been reported in grains. Moore *et al* determined the free, soluble conjugated and insoluble bound phenolic acid contents of eight Maryland-grown soft wheat varieties. Total vanillic acid content ranged from 8.4 μg/g (SS560) to 12.7 μg/g (Roane) (*53*). Total syringic acid content ranged from 8.9 μg/g (MV5-46) to 17.8 μg/g (Choptank). Total p-coumaric acid content ranged from 10.4 μg/g (VA97W-024) to 14.1 μg/g (McCormick). In all cases, most of the phenolic acid content was found in the insoluble bound fraction (*53*).

Matilla *et al* reported the phenolic acid content of commonly consumed grain products. Rye bran and wheat bran had the highest amount of ferulic acid, 2800 ± 150 and 3000 ± 180 mg/kg, respectively. Whole grain rye flour and whole-wheat flour also had similarly high amounts of ferulic acid, 860 ± 71, 860 ± 79, and 890 ± 40 mg/kg, respectively (*54*). Most likely, the bulk of the ferulic acid in those whole-grain flour samples came from the bran, as the data shows that white wheat flour and organic white wheat flour have ferulic acid contents of only 120 ± 12 and 100 ± 7 mg/kg, respectively (*54*).

1.4.1.3 Alk(en)ylresorcinols

Alkylresorcinols and alkenylresorcinols are amphiphillic derivatives of 1,3 dihydroxybenzene with an odd-numbered alkyl or alkenyl chain at position 5 of the benzene ring (**Figure 1.6**). They are generally found in the bran fraction of the grain and, for this reason, are missing in refined grains (*55*).

Ross *et al* reported the levels of alkylresorcinols in grains. Rye had the most total alkylresorcinol 734 ± 23 µg/g DW. Wheat had a total alkylresorcinol content of 583 ± 82 µg/g DW. Barley had a total alkylresorcinol content of 45 ± 5 µg/g DW (*55*). Rye was the only grain to have detectable amounts of the 15-carbon alkylresorcinol homologue. The 19 and 21 carbon homologues were prominent in wheat. The 25-carbon homologue was prominent in barley (*55*).   No alkylresorcinols were detected in corn, millet, oats, rice, and sorghum.

Mattila *et al* determined the amount of alkylresorcinols in whole grains. Of the cereal products analyzed, alkylresorcinol content ranged from 32 mg/kg FW (whole grain barley flour) to 4108 mg/kg FW (whole grain rye bread) (*54*). Alkylresorcinols were not detected in any oat products, whole grain buckwheat grits, millet grits, long grain parboiled rice and corn grits. The only alkenylresorcinol detected in these products was the 19:1 homologue. Content of this alkenylresorcinol ranged from 13 mg/kg FW (organic and conventional white wheat flour) to 130 ± 14 mg/kg FW (rye bran) (*54*).

Landberg *et al* reported the total alkylresorcinol content of durum wheat grown in different countries. Whole wheat from Kazakstan had the most total alkylresorcinol content (554 ± 65 µg/g DW), followed by Spain (473 ± 65 µg/g DW), Sweden (471 ± 37 µg/g DW) and Austria (389 ± 72 µg/g DW) (**Figure 1.7**) (*56*). The 21 carbon homologue accounted for roughly 60% of the total alkylresorcinol content in all cases. These results suggest that growing conditions can affect the total alkylresorcinol content of wheat.

1.4.1.4 Flavonoids

Flavonoids are phenolic compounds consisting of two aromatic rings joined by a three-carbon structure generally found in an oxygenated heterocyclic ring. The
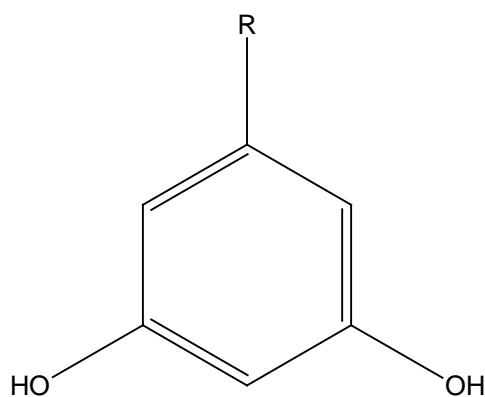
Name	R	Structural Formula
5-n-pentadecylresorcinol	$C_{15}H_{31}$	$CH_3\text{-}(CH_2)_3\text{-}CH=CH\text{-}(CH_2)_8\text{-}CH_2\text{-}$
5-n-heptadecylresorcinol	$C_{17}H_{35}$	$CH_3\text{-}(CH_2)_3\text{-}CH=CH\text{-}(CH_2)_{10}\text{-}CH_2\text{-}$
5-n-nonadecylresorcinol	$C_{19}H_{39}$	$CH_3\text{-}(CH_2)_3\text{-}CH=CH\text{-}(CH_2)_{12}\text{-}CH_2\text{-}$
5-n-heneicosylresorcinol	$C_{21}H_{43}$	$CH_3\text{-}(CH_2)_3\text{-}CH=CH\text{-}(CH_2)_{14}\text{-}CH_2\text{-}$
5-n-tricosylresorcinol	$C_{23}H_{47}$	$CH_3\text{-}(CH_2)_3\text{-}CH=CH\text{-}(CH_2)_{16}\text{-}CH_2\text{-}$

Figure 1.6 – Structure of alkylresorcinols.
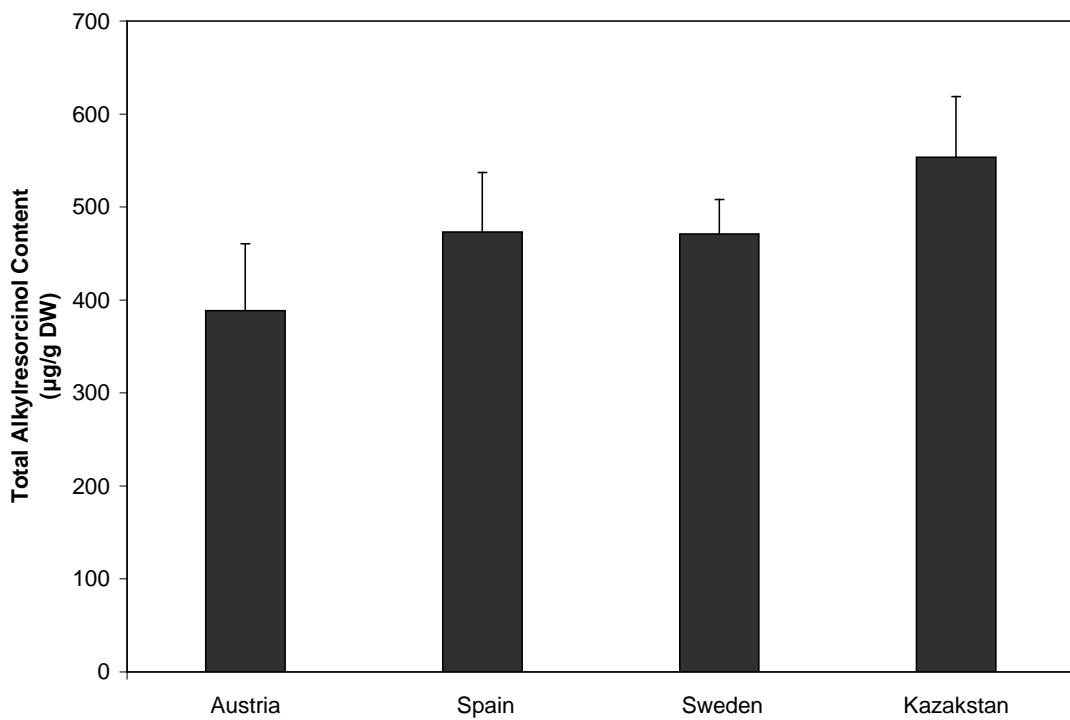
Figure 1.7 – Total alkylresorinol content of Durum wheat from different countries. Data obtained from Landberg *et al* (*56*).

differences in the structure of the heterocyclic ring determine the class of flavonoid (**Figure 1.8**). Flavonoids are usually found as conjugates in glycosylated or esterified forms and may account for as much as two thirds of the phenolic content of the North American diet (*48*). Flavonoids have potent antioxidant activity, and are also linked to reduced risk of major chronic diseases.

Adom *et al* reported the flavonoid content of 11 diverse wheat varieties. Total flavonoid content ranged from 122 ± 10 μmol catechin equivalents/g (Roane) to 149 ± 17 μmol catechin equivalents/g (Superior) (*57*). Most of the flavonoid content was located in the bound fraction of the grain ranging from 970 ± 4 μmol catechin equivalents/g (Roane) to 139 ± 17 μmol catechin equivalents/g (Superior). Adom et al also reported that the majority of total flavonoids of whole wheat grains were present in the bran/germ fraction (*52*). Similar to total phenolics, in whole grain wheat flour, the bran/germ fraction contributed 79% of total flavonoid content (*52*).

Intake of foods with significant amounts of flavonoid has been associated with health benefits. Naderi *et al* showed that dietary intake of flavonoid has been shown to be inversely associated with risk of coronary artery disease (*58*). Further, Duthie *et al* (2000) showed that flavonoids have the ability to prevent oxidation of LDL (*59*). This prevention of oxidation may be due to the finding that some flavonoids have the ability to chelate iron and copper (*60*), two metals that are important in the generation of hydroxyl radical, or the combination of a conjugated ring structure and hydroxyl groups that allows the compound to scavenge a free radical from singlet oxygen.

1.4.2 Carotenoids

Carotenoids, natural pigments of fruits, vegetables, and whole grains, are compounds consisting of a forty-carbon skeleton. These compounds are usually found in the all-trans form in nature. Carotenoids can be cyclized at one or both ends of the
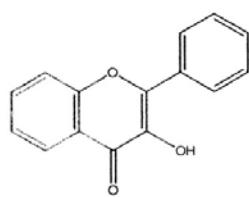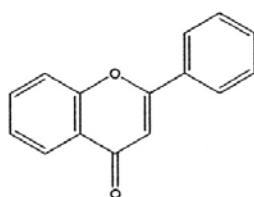
Flavonols   Flavones   Flavanols (Catechins)
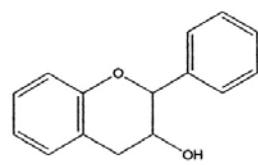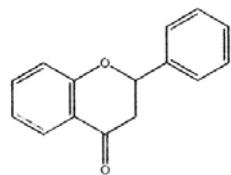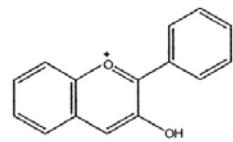
Flavanones   Anthocyanidins   Isoflavonoids
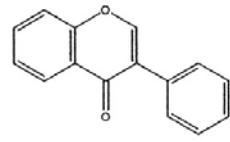
Figure 1.8 – Structure of main classes of dietary flavonoids (*48*).

structure and may be hydrogenated to different degrees. Carotenoids can also have oxygen-containing functional groups (**Figure 1.9**). One of the most characteristic features of carotenoids is the long series of alternating double and single bonds. This characteristic makes carotenoids very good antioxidants. Carotenoids can scavenge free radicals, becoming free radicals themselves in the process, and remain stable compounds because of their ability to delocalize the free radical amongst its alternating double and single bonds.

Adom *et al* reported the carotenoid (lutein, zeazanthin, and β-cryptoxanthin) content of 11 diverse wheat varieties and experimental lines (*57*). β-Cryptoxanthin content from 1.1 ± 0.1 µg/g (W7985) to 13.3 ± 0.3 µg/g (Stoa). Zeaxanthin content from 8.7 ± 0.8 µg/g (Cham1) to 27.1 ± 0.5 µg/g (Superior). Lutein content ranged from 26.4 ± 1.4 µg/g (W7985) to 143.5 ± 6.7 µg/g (Roane).

Brenna and Berardo reported the carotenoid content of corn using Near-Infrared Reflectance Spectroscopy (NIRS). β-Cryptoxanthin content of the 40 corn flour samples was 3.7 ± 0.2 mg/kg, lutein content was 11.5 ± 0.8 mg/kg, and zeaxanthin content was 17.5 ± 1.7 mg/kg (*61*).

Miller *et al* reported that β-carotene and lycopene were responsible for anticarcinogenesis in fresh fruits and vegetables (*62*). Mannisto *et al* (*63*) investigated the relationship between dietary carotenoids and risk of colorectal cancer. There was no association between lycopene intake and risk of colorectal cancer in studies that included tomato sauce consumption in the food frequency questionnaire (RR = 1.08; 95% CI = 0.98 – 1.20) (*63*). When pooling the data, there was no association between intake of β-carotene and relative risk of colorectal cancer in subjects aged less than 65 years (RR = 1.08; 95% CI = 0.91 – 1.28) or subjects aged 65 years or more (RR = 0.90; 95% CI = 0.79 – 1.02) (*63*). Lutein and zeaxanthin intakes greater than 4000 µg per day, an amount equal to roughly half of a pound of broccoli, was associated with
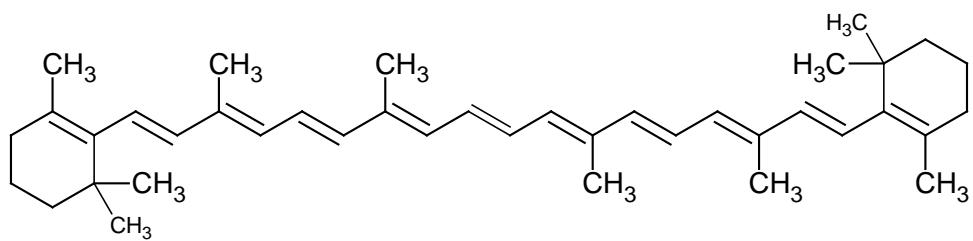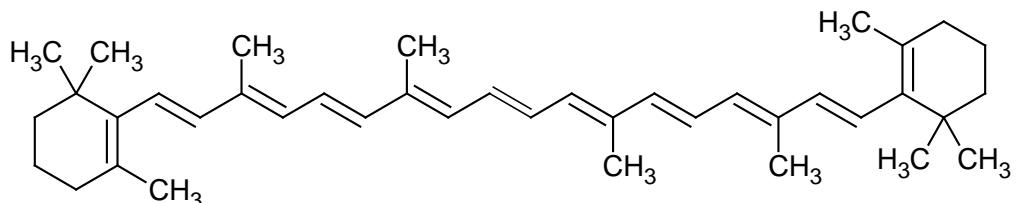
α-Carotene
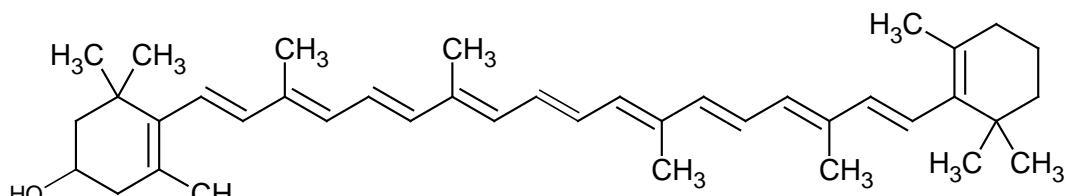
β-Carotene

β-Cryptoxanthin
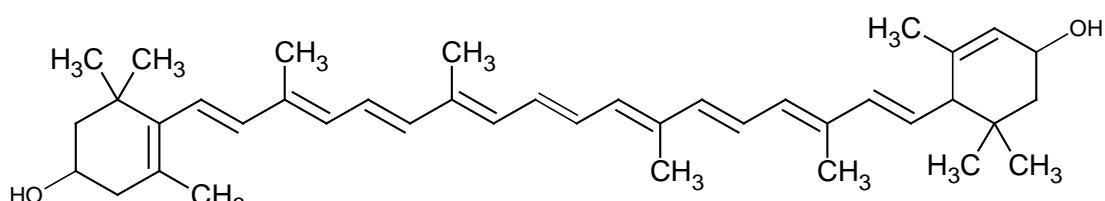
Lutein
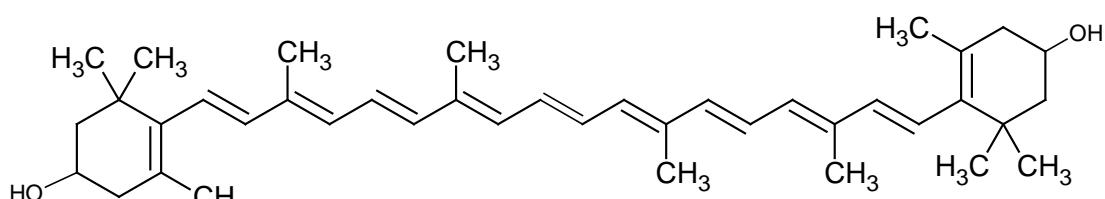
Zeaxanthin

Figure 1.9 – Structure of carotenoids.

reduced risk of colorectal cancer (RR = 0.87; 95% CI = 0.78 – 0.98) when compared to a lutein and zeaxanthin intake of less than 1000 μg per day (*63*).

1.4.3 Vitamin E

Vitamin E is a collective name for eight lipid-soluble compounds consisting of a chromanol ring and a phytyl tail (**Figure 1.10**). Tocopherols refer to the vitamin E compounds with a fully saturated phytyl tail and tocotrienols refer to the vitamin E compounds with a polyunsaturated phytyl tail. Both tocopherols and tocotrienols vary in the degree of methylation of the chromanol ring, with four vitamers each; α, β, γ, and δ.

Panfili *et al* determined the total vitamin E content and vitamer distribution of several whole grains. Barley and soft wheat had the most total vitamin E, 75 ± 13 mg/kg DW and 74 mg/kg DW, respectively (*64*). Spelt had the lowest overall vitamin E content with 57 ± 8 mg/kg DW. Barley was the only grain to have all eight vitamers of vitamin E. Spelt, durum wheat, soft wheat, and triticale had the fewest number of vitamers, five, even although soft wheat had the second highest total vitamin E content. All six grains contained α-tocopherol. Corn had the least amount of α-tocopherol (4 mg/kg DW) while soft wheat had the most α-tocopherol (16 ± 2 mg/kg DW). β-Tocotrienol was the predominant vitamer in soft wheat, triticale, and spelt, followed by α-tocopherol, β-tocopherol and α-tocotrienol. α-Tocotrienol was the predominant vitamer in oats, followed by α-tocopherol, β-tocotrienol, β-tocopherol, and γ-tocopherol. γ-Tocopherol was the predominant vitamer in corn, followed by γ-tocotrienol, α-tocopherol, and β-tocotrienol. Oats, corn, and barley were the only grains that contained γ-tocopherol while corn and barley were the only grains that contained γ-tocotrienol. Corn and barley had similar γ-tocotrienol content, with 11 and 10 ± 3 mg/kg DW, respectively (*64*).
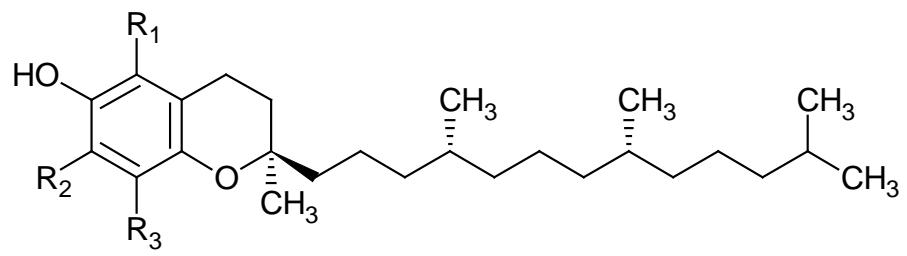
Tocopherols
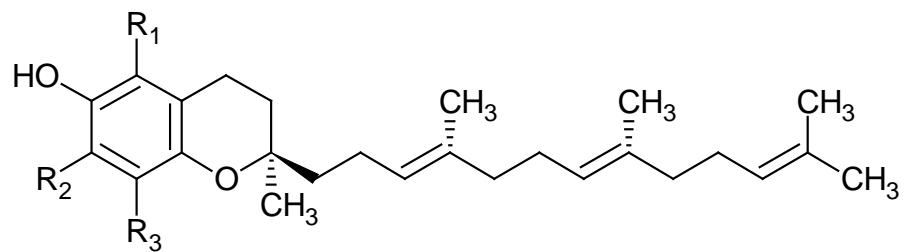
Tocotrienols

	R$_1$	R$_2$	R$_3$
α	CH$_3$	CH$_3$	CH$_3$
β	CH$_3$	H	CH$_3$
γ	H	CH$_3$	CH$_3$
δ	H	H	CH$_3$

Figure 1.10 – Structure of vitamin E: tocopherols and tocotrienols.

In similar studies investigating the content of vitamin E in whole grains, Hakkarainen *et al* did not report the presence of γ-tocotrienol in barley (*65*), while Grela reported γ-tocopherol in both spelt and durum wheat (*66*). This variation in quantities and presence of the various forms of vitamin E may be due to a lack of standardized methods for extraction and analysis (*64*).

Vitamin E and its derivatives, mainly α-tocopheryl succinate, have been reported to have health benefits, including inhibition of lipoxygenase activity *in vivo*, induction of apoptosis in prostate cancer cells via inhibition of Bcl-2, and protection against photo-inflammation when applied on the skin (*67-69*). Recently, using data from the Women's Health Study, Liu *et al* reported that there is an inverse association between vitamin E consumption and risk of type II diabetes in women who had no familial history of type II diabetes (RR = 0.88, 95% CI = 0.78 – 1.00) (*70*). However, there was no significant association between vitamin E and the development of type II diabetes for all women (RR = 0.95, 95% CI = 0.87 – 1.05).

1.4.4 γ-Oryzanol

γ-Oryzanols are compounds that consist of a phenolic acid esterified to a sterol. Common γ-oryzanol compounds include cycloartenyl ferulate, 24-methylenecycloartanylferulate, and campesteryl ferulate (**Figure 1.11**).

Generally, γ-oryzanol is found in rice, particularly in the bran fraction, and γ-oryzanol content is around 3000 mg/kg of rice (*71*). γ-Oryzanols have also been identified in wheat bran and rye bran. In wheat bran, γ-oryzanol content ranged from 300 – 390 mg/kg (*72*). One of the main differences between the γ-oryzanols found in rice and in wheat or rye is that the sterols in rice are dimethyl sterols, with two methyl groups on the fourth carbon of the molecule in rice, whereas the γ-oryzanols in wheat or rye do not have the two methyl groups (*73*).
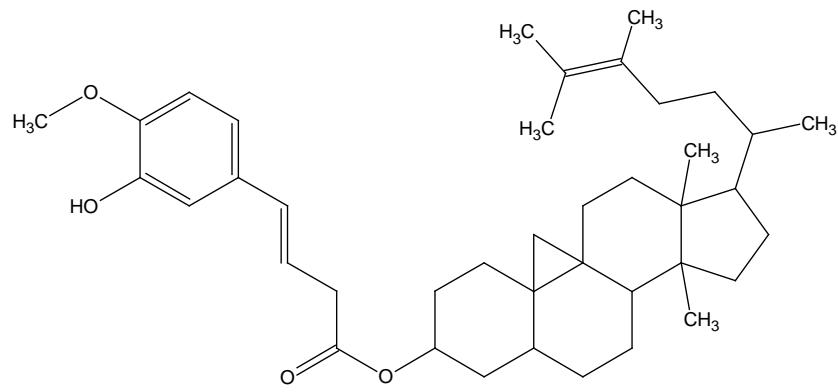
Cycloartenyl Ferulate
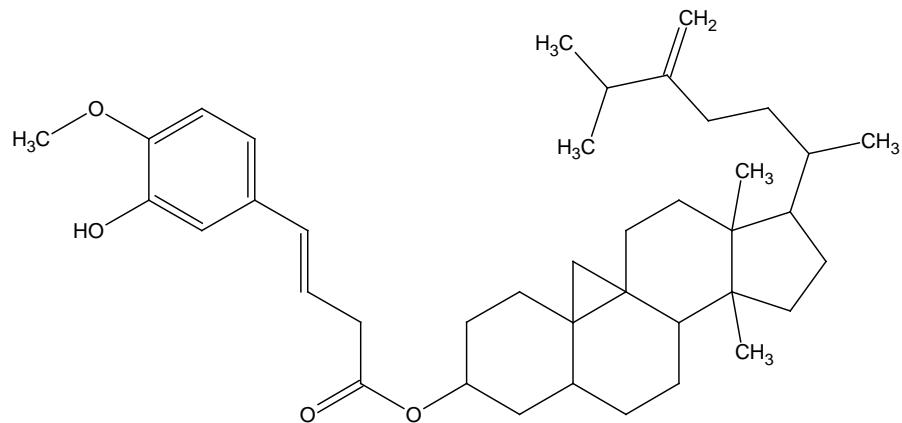
24-Methylene Cycloartanyl Ferulate
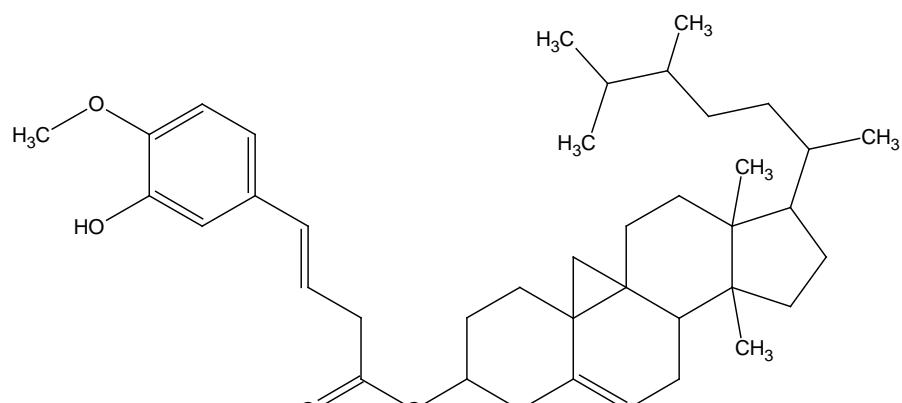
Campesteryl Ferulate

Figure 1.11 – Structure of γ-oryzanols

γ-Oryzanol has been shown to have antioxidant activity and serum cholesterol lowering effects. Xu *et al* conducted a study using ABAP to initiate the oxidation of cholesterol and assessed the antioxidant potential of γ-oryzanols and vitamin E. The study showed that γ-oryzanols were more effective than tocopherols and tocotrienols in the prevention of cholesterol oxidation (*74*). Other γ-oryzanols, such as cycloartenyl ferulate and 24-methylenecycloartanyl ferulate, were shown to be radical scavengers in multiphase lipid systems (*75*) and, in the case of campesteryl ferulate, to be inhibitors of UV irradiation-initiated linoleic acid oxidation (*76*). Trautwein *et al* also reported that dimethylsterols were as effective in lowering plasma total cholesterol and LDL cholesterol as dimethylstanols, although esterified dimethylsterols had a lesser effect (*77*).

Other studies have shown the cholesterol-lowering properties of γ-oryzanol, specifically in rats fed a high cholesterol diet with varying amounts of γ-oryzanol (*78*). Although previous studies have investigated the cholesterol-lowering effect of rice bran oil in a population of schizophrenics and mixed clinical populations including hyperlipidemic men, few studies have looked specifically at the cholesterol-lowering properties of γ-oryzanols in humans (*79, 80*). In a clinical trial consisting of hypercholesterolemic men, Berger *et al* (*81*) investigated the cholesterol-lowering properties of rice bran oils containing different amount of γ-oryzanols. LDL cholesterol decreased by 12% two weeks after the change from a peanut oil-based diet containing no γ-oryzanol to a rice bran oil-based diet containing γ-oryzanol (*81*). Further, the ratio of LDL cholesterol to HDL cholesterol decreased by 19% in four weeks following the change from the peanut oil-based diet to the rice bran oil-based diet (*81*). As the authors noted, other bioactive compounds such as unsaturated fatty acids, tocotrienols, and ferulic acid may had contributed to the cholesterol-lowering effects of rice bran oil consumption besides γ-oryzanol (*81*).

1.4.5 Dietary Fiber

Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine and are completely or partially fermented in the large intestine (*82*). Major components of dietary fiber include lignin, hemicellulose, cellulose, and β-glucan. Consumption of dietary fiber has been associated with reduced risk of CHD, type II diabetes, major weight gain, and colorectal cancer (*5, 21, 30, 83*).

Lignin is an aromatic polymer formed from the condensation of coniferyl, sinapyl, and p-hydroxycimamyl alcohols. This condensation does not require enzymes and, for that reason, the polymerization can occur at random. The process of polymerization of lignin compounds can lead to hydrophobic regions in the plant cell wall. Lignin is found in the cell wall of plants and can form complexes with cellulose and hemicelluloses. Lignin is extremely resistant to degradation, remaining insoluble in 12 M sulfuric acid (*84*).

Lignin's chemical heterogeneity, resistance to chemical reaction, conjugation to starches, and uneven and low distribution in plants makes the extraction and analysis of lignin particularly difficult. The Klason procedure is the most commonly used procedure for measuring lignin content and requires the removal of all other compounds in the cell wall with concentrated sulfuric acid followed by gravimetric measurement (*84*). However, the Klason procedure does have some limitations. Some lignin is lost in the acid hydrolysis step because lignin is slightly soluble in some lipid solvents. Further, this procedure does not remove cutin and suberin, which can lead to the overestimation of lignin content (*85*). Lignin content can also be determined from acid detergent fibers using the procedure developed by Van Soest and Wine (*86*).

Hemicellulose is a group of water-insoluble hetero-polysaccharides containing pentoses and hexoses. The true chemical nature of hemicelluloses has been difficult to determine due to a lack of uniform extraction, purification, and analytical procedures. Cellulose is a linear polymer of glucose with β-(1,4)-linkages. Due to its linearity and hydrogen bonding between chains, cellulose is highly insoluble and can be crystalline in structure. Sun *et al* (2005) investigated the structure of original lignins and hemicelluloses from wheat straw. The hemicellulose consisted mainly of six sugars, rhamnose, arabinose, xylose, mannose, galactose, and glucose (*87*).

β-Glucans are polysaccharides composed of glucopyranosyl units (*88*). In grains, β-glucans are linear molecules consisting of 30% of (1→3) and 70% of (1→4) β linkages. Compared to cellulose with only β-(1-4)-linkages, the β-(1-3)–linkages interrupt β-(1-4)–linkages to make beta-glucan more flexible, soluble, and viscous. These β-glucans are components of the endosperm cell walls.

The health benefits of β-glucan include lowering of serum cholesterol level and controlling blood sugar (*89, 90*). These health benefits are attributed to viscosity of β-glucan in the gastrointestinal tract. The concentration and molecular weight of the β-glucan polymer affect this viscosity. Because β-glucan is a compound that is characteristic of oats and barley, it can be considered as a marker for whole oats or whole barley. Braaten *et al* reported that β-glucan in oat products was responsible for the 10% decline in LDL cholesterol concentrations in hyperchlosterolemic men and women who ate a diet consisting of 5.8 g daily of β-glucan for four weeks (*91*). This amount of β-glucan would equal roughly 70 g of oat bran per day. The FDA has approved the health claim about β-glucan that 3 g per day of β-glucan from oats can be eaten for a clinically relevant reduction in serum total cholesterol concentration (*92*). Mackay and Ball did not see a reduction in plasma LDL cholesterol concentration in hypercholesterolemic men and women who consumed a low-fat diet with roughly 3 g

daily of β-glucan for six weeks, although they did notice an increase in plasma HDL cholesterol concentration (*93*). β-Glucan also had an effect in controlling blood sugar in diabetes subjects, and was helpful in reducing the elevation in blood sugar levels after a meal (*91*), probably by delay of gastric emptying, allowing dietary sugar to be absorbed more gradually, or by increasing the tissue sensitivity to insulin.

1.5 Conclusions

A whole grain consists of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components - the starchy endosperm, germ and bran - are present in the same relative proportions as they exist in the intact caryopsis (*14*). Whole grain products can be intact, having the original composition of bran, germ, and endosperm throughout the lifetime of the product or reconstituted, in which one or more of the original components are added back to the product to achieve the relative proportions found in nature. Whole grain consumption is associated with reduced risk of chronic diseases including cardiovascular disease, type II diabetes, and some cancers (*20, 23, 34, 43, 44*). Thus, the Dietary Guidelines for Americans recommends that Americans consume at least 3 ounce-equivalents of whole grain each day (*10*). The health benefit of whole grain consumption may be due to their unique phytochemicals. Whole grain phytochemicals and bioactive compounds including phenolics, carotenoids, vitamin E, γ-oryzanol, dietary fiber, and β-glucan may be responsible for the health benefits of whole grain consumption in the prevention of chronic diseases.

LITERATURE CITED

1.   Fraser, G. E., Associations between diet and cancer, ischemic heart disease, and all-cause mortality in non-Hispanic white California Seventh-day Adventists. *Am J Clin Nutr* **1999,** 70, (3 Suppl), 532S-538S.

2.   Jacobs, D. R., Jr.; Meyer, K. A.; Kushi, L. H.; Folsom, A. R., Whole-grain intake may reduce the risk of ischemic heart disease death in postmenopausal women: the Iowa Women's Health Study. *Am J Clin Nutr* **1998,** 68, (2), 248-57.

3.   Liu, S.; Stampfer, M. J.; Hu, F. B.; Giovannucci, E.; Rimm, E.; Manson, J. E.; Hennekens, C. H.; Willett, W. C., Whole-grain consumption and risk of coronary heart disease: results from the Nurses' Health Study. *Am J Clin Nutr* **1999,** 70, (3), 412-9.

4.   Liu, S.; Manson, J. E.; Stampfer, M. J.; Hu, F. B.; Giovannucci, E.; Colditz, G. A.; Hennekens, C. H.; Willett, W. C., A prospective study of whole-grain intake and risk of type 2 diabetes mellitus in US women. *Am J Public Health* **2000,** 90, (9), 1409-15.

5.   Meyer, K. A.; Kushi, L. H.; Jacobs, D. R., Jr.; Slavin, J.; Sellers, T. A.; Folsom, A. R., Carbohydrates, dietary fiber, and incident type 2 diabetes in older women. *Am J Clin Nutr* **2000,** 71, (4), 921-30.

6.   Salmeron, J.; Ascherio, A.; Rimm, E. B.; Colditz, G. A.; Spiegelman, D.; Jenkins, D. J.; Stampfer, M. J.; Wing, A. L.; Willett, W. C., Dietary fiber, glycemic load, and risk of NIDDM in men. *Diabetes Care* **1997,** 20, (4), 545-50.

7.   Jacobs, D. R., Jr.; Slavin, J.; Marquart, L., Whole grain intake and cancer: a review of the literature. *Nutr Cancer* **1995,** 24, (3), 221-9.

8.   Smigel, K., Fewer colon polyps found in men with high-fiber, low-fat diets. *J Natl Cancer Inst* **1992,** 84, (2), 80-1.

9.   USDA *Nutrition and Your Health: Dietary Guidelines for Americans*; USDA Human Nutrition Information Service: Hyattsville, MD, 1995.

10.   USDA *Dietary Guidelines for Americans 2005*; USDA Human Nutrition Information Service Hyattsville, MD, 2005.

11.   Burros, M., Whole Grain Labels Become More Transparent. *New York Times* 2006.

12.   Vocke, G.; Allen, E. *Wheat Situation and Outlook Yearbook* USDA Economic Research Service - Market and Trade Economics Division 2007.

13.   WGC *Whole Grain Facts and Figures.* ; Whole Grain Council Facts and Figures http://www.wholegrainscouncil.org/WGFacts+Figures.htm: 2007

14.   AACC *AACC Members Agree on Definition of Whole Grain*; AACC St. Paul, MN 1999.

15.   Distaam, E. M.; Carcea, M., Pasta from nontraditional raw materials *Cereal Foods World* **2001,** 46, 522-530.

16.   Seib, P. A.; Liang, X.; Guan, F.; Liang, Y. T.; Yang, H. C., Comparison of Asian noodles from some hard white and hard red wheat flours. *Cereal Chemistry* **2000,** 77, (6), 816-822.

17.   Pietinen, P.; Rimm, E. B.; Korhonen, P.; Hartman, A. M.; Willett, W. C.; Albanes, D.; Virtamo, J., Intake of dietary fiber and risk of coronary heart disease in a cohort of Finnish men. The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. *Circulation* **1996,** 94, (11), 2720-7.

18.   Wolk, A.; Manson, J. E.; Stampfer, M. J.; Colditz, G. A.; Hu, F. B.; Speizer, F. E.; Hennekens, C. H.; Willett, W. C., Long-term intake of dietary fiber and decreased risk of coronary heart disease among women. *Jama* **1999,** 281, (21), 1998-2004.

19.   Beeson, W. L.; Mills, P. K.; Phillips, R. L.; Andress, M.; Fraser, G. E., Chronic disease among Seventh-day Adventists, a low-risk group. Rationale, methodology, and description of the population. *Cancer* **1989,** 64, (3), 570-81.

20.   Anderson, J. W.; Hanna, T. J.; Peng, X.; Kryscio, R. J., Whole grain foods and heart disease risk. *J Am Coll Nutr* **2000,** 19, (3 Suppl), 291S-299S.

21.   Pereira, M. A.; O'Reilly, E.; Augustsson, K.; Fraser, G. E.; Goldbourt, U.; Heitmann, B. L.; Hallmans, G.; Knekt, P.; Liu, S.; Pietinen, P.; Spiegelman, D.; Stevens, J.; Virtamo, J.; Willett, W. C.; Ascherio, A., Dietary fiber and risk of coronary heart disease: a pooled analysis of cohort studies. *Arch Intern Med* **2004,** 164, (4), 370-6.

22.   Erkkila, A. T.; Herrington, D. M.; Mozaffarian, D.; Lichtenstein, A. H., Cereal fiber and whole-grain intake are associated with reduced progression of coronary-artery atherosclerosis in postmenopausal women with coronary artery disease. *American Heart Journal* **2005,** 150, (1), 94-101.

23.   Lockheart, M. S.; Steffen, L. M.; Rebnord, H. M.; Fimreite, R. L.; Ringstad, J.; Thelle, D. S.; Pedersen, J. I.; Jacobs, D. R., Jr., Dietary patterns, food groups and myocardial infarction: a case-control study. *Br J Nutr* **2007,** 98, (2), 380-7.

24.   Wang, L.; Gaziano, J. M.; Liu, S. M.; Manson, J. E.; Buring, J. E.; Sesso, H. D., Whole- and refined-grain intakes and the risk of hypertension in women. *American Journal of Clinical Nutrition* **2007,** 86, (2), 472-479.

25.   Lutsey, P. L.; Jacobs, D. R.; Kori, S.; Mayer-Davis, E.; Shea, S.; Steffen, L. M.; Szklo, M.; Tracy, R., Whole grain intake and its cross-sectional association with obesity, insulin resistance, inflammation, diabetes and subclinical CVD: The MESA study. *British Journal of Nutrition* **2007,** 98, (2), 397-405.

26.   Flint, A. J.; Hu, F. B.; Glynn, R. J.; Jensen, M. K.; Franz, M.; Sampson, L.; Rimm, E. B., Whole grains and incident hypertension in men. *Am J Clin Nutr* **2009,** 90, (3), 493-8.

27.   Nettleton, J. A.; Polak, J. F.; Tracy, R.; Burke, G. L.; Jacobs, D. R., Jr., Dietary patterns and incident cardiovascular disease in the Multi-Ethnic Study of Atherosclerosis. *Am J Clin Nutr* **2009,** 90, (3), 647-54.

28.   van Dam, R. M.; Rimm, E. B.; Willett, W. C.; Stampfer, M. J.; Hu, F. B., Dietary patterns and risk for type 2 diabetes mellitus in U.S. men. *Ann Intern Med* **2002,** 136, (3), 201-9.

29.   Fung, T. T.; Hu, F. B.; Pereira, M. A.; Liu, S.; Stampfer, M. J.; Colditz, G. A.; Willett, W. C., Whole-grain intake and the risk of type 2 diabetes: a prospective study in men. *Am J Clin Nutr* **2002,** 76, (3), 535-540.

30.   Liu, S.; Willett, W. C.; Manson, J. E.; Hu, F. B.; Rosner, B.; Colditz, G., Relation between changes in intakes of dietary fiber and grain products and changes in weight and development of obesity among middle-aged women. *Am J Clin Nutr* **2003,** 78, (5), 920-7.

31.   Montonen, J.; Knekt, P.; Jarvinen, R.; Aromaa, A.; Reunanen, A., Whole-grain and fiber intake and the incidence of type 2 diabetes. *Am J Clin Nutr* **2003,** 77, (3), 622-629.

32.   Schulze, M. B.; Liu, S. M.; Rimm, E. B.; Manson, J. E.; Willett, W. C.; Hu, F. B., Glycemic index, glycemic load, and dietary fiber intake and incidence of type 2 diabetes in younger and middle-aged women. *American Journal of Clinical Nutrition* **2004,** 80, (2), 348-356.

33.   Bazzano, L. A.; Song, Y. Q.; Bubes, V.; Good, C. K.; Manson, J. E.; Liu, S. M., Dietary intake of whole and refined grain breakfast cereals and weight gain in men. *Obesity Research* **2005,** 13, (11), 1952-1960.

34.   Munter, J. S. L. d.; Hu, F. B.; Spiegelman, D.; Franz, M.; Dam, R. M. v., Whole grain, bran, and germ intake and risk of type 2 diabetes: a prospective cohort study and systematic review. *PLoS Medicine* **2007,** 4, (8), e261.

35.   Newby, P. K.; Maras, J.; Bakun, P.; Muller, D.; Ferrucci, L.; Tucker, K. L., Intake of whole grains, refined grains, and cereal fiber measured with 7-d diet records and associations with risk factors for chronic disease. *American Journal of Clinical Nutrition* **2007,** 86, (6), 1745-1753.

36.   Nettleton, J. A.; Steffen, L. M.; Ni, H.; Liu, K.; Jacobs, D. R., Jr., Dietary patterns and risk of incident type 2 diabetes in the Multi-Ethnic Study of Atherosclerosis (MESA). *Diabetes Care* **2008,** 31, (9), 1777-82.

37.   Rohan, T. E.; Hiller, J. E.; McMichael, A. J., Dietary factors and survival from breast cancer. *Nutr Cancer* **1993,** 20, (2), 167-77.

38.   Nicodemus, K. K.; Jacobs, D. R., Jr.; Folsom, A. R., Whole and refined grain intake and risk of incident postmenopausal breast cancer (United States). *Cancer Causes Control* **2001,** 12, (10), 917-25.

39.   Willett, W. C.; Hunter, D. J.; Stampfer, M. J.; Colditz, G.; Manson, J. E.; Spiegelman, D.; Rosner, B.; Hennekens, C. H.; Speizer, F. E., DIETARY-FAT AND FIBER IN RELATION TO RISK OF BREAST-CANCER - AN 8-YEAR FOLLOW-UP. *Jama-Journal of the American Medical Association* **1992,** 268, (15), 2037-2044.

40.   Pietinen, P.; Malila, N.; Virtanen, M.; Hartman, T. J.; Tangrea, J. A.; Albanes, D.; Virtamo, J., Diet and risk of colorectal cancer in a cohort of Finnish men. *Cancer Causes Control* **1999,** 10, (5), 387-96.

41.   Kasum, C. M.; Nicodemus, K.; Harnack, L. J.; Jacobs, D. R.; Folsom, A. R., Whole grain intake and incident endometrial cancer: The Iowa Women's Health Study. *Nutrition and Cancer-an International Journal* **2001,** 39, (2), 180-186.

42.   Adlercreutz, H.; Mazur, W., Phyto-oestrogens and Western diseases. *Ann Med* **1997,** 29, (2), 95-120.

43.   Larsson, S. C.; Giovannucci, E.; Bergkvist, L.; Wolk, A., Whole grain consumption and risk of colorectal cancer: a population-based cohort of 60 000 women. *British Journal of Cancer* **2005,** 92, (9), 1803-1807.

44.   Schatzkin, A.; Mouw, T.; Park, Y.; Subar, A. F.; Kipnis, V.; Hollenbeck, A.; Leitzmann, M. F.; Thompson, F. E., Dietary fiber and whole-grain consumption in relation to colorectal cancer in the NIH-AARP Diet and Health Study. *Am J Clin Nutr* **2007,** 85, (5), 1353-60.

45.   Chan, J. M.; Wang, F.; Holly, E. A., Whole grains and risk of pancreatic cancer in a large population-based case-control study in the San Francisco Bay Area, California. *Am J Epidemiol* **2007,** 166, (10), 1174-85.

46.   Sonestedt, E.; Borgquist, S.; Ericson, U.; Gullberg, B.; Landberg, G.; Olsson, H.; Wirfalt, E., Plant foods and oestrogen receptor alpha- and beta-defined breast cancer: observations from the Malmo Diet and Cancer cohort. *Carcinogenesis* **2008,** 29, (11), 2203-9.

47.   Williams, C. D.; Satia, J. A.; Adair, L. S.; Stevens, J.; Galanko, J.; Keku, T. O.; Sandler, R. S., Dietary patterns, food groups, and rectal cancer risk in Whites and African-Americans. *Cancer Epidemiol Biomarkers Prev* **2009,** 18, (5), 1552-61.

48.   Liu, R. H., Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J Nutr* **2004,** 134, (12 Suppl), 3479S-3485S.

49.   Zielinski, H.; Kozlowska, H., Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. *Journal of Agricultural and Food Chemistry* **2000,** 48, (6), 2008-2016.

50.   Adom, K. K.; Liu, R. H., Antioxidant activity of grains. *J Agric Food Chem* **2002,** 50, (21), 6182-7.

51.   Sosulski, F.; Krygier, K.; Hogge, L., Free, Esterified, and Insoluble-Bound Phenolic-Acids .3. Composition of Phenolic-Acids in Cereal and Potato Flours. *Journal of Agricultural and Food Chemistry* **1982,** 30, (2), 337-340.

52.   Adom, K. K.; Sorrells, M. E.; Liu, R. H., Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. *J Agric Food Chem* **2005,** 53, (6), 2297-306.

53.   Moore, J.; Hao, Z.; Zhou, K.; Luther, M.; Costa, J.; Yu, L. L., Carotenoid, tocopherol, phenolic acid, and antioxidant properties of Maryland-grown soft wheat. *J Agric Food Chem* **2005,** 53, (17), 6649-57.

54.   Mattila, P.; Pihlava, J. M.; Hellstrom, J., Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. *J Agric Food Chem* **2005,** 53, (21), 8290-5.

55.   Ross, A. B.; Shepherd, M. J.; Schupphaus, M.; Sinclair, V.; Alfaro, B.; Kamal-Eldin, A.; Aman, P., Alkylresorcinols in cereals and cereal products. *Journal of Agricultural and Food Chemistry* **2003,** 51, (14), 4111-4118.

56.   Landberg, R.; Kamal-Eldin, A.; Andersson, R.; Aman, P., Alkylresorcinol content and homologue composition in durum wheat (Triticum durum) kernels and pasta products. *J Agric Food Chem* **2006,** 54, (8), 3012-4.

57.   Adom, K. K.; Sorrells, M. E.; Liu, R. H., Phytochemical profiles and antioxidant activity of wheat varieties. *J Agric Food Chem* **2003,** 51, (26), 7825-34.

58.   Naderi, G. A.; Asgary, S.; Sarraf-Zadegan, N.; Shirvany, H., Anti-oxidant effect of flavonoids on the susceptibility of LDL oxidation. *Mol Cell Biochem* **2003,** 246, (1-2), 193-6.

59.   Duthie, G. G.; Duthie, S. J.; Kyle, J. A. M., Plant polyphenols in cancer and heart disease: Implications as nutritional antioxidants *Nutr. Res. Rev.* **2000,** 13, 79-106.

60.   Yao, L. H.; Jiang, Y. M.; Shi, J.; Tomas-Barberan, F. A.; Datta, N.; Singanusong, R.; Chen, S. S., Flavonoids in food and their health benefits. *Plant Foods Hum Nutr* **2004,** 59, (3), 113-22.

61.   Brenna, O. V.; Berardo, N., Application of near-infrared reflectance spectroscopy (NIRS) to the evaluation of carotenoids content in maize. *J Agric Food Chem* **2004,** 52, (18), 5577-82.

62.   Miller, E. C.; Hadley, C. W.; Schwartz, S. J.; Erdman, J. W.; Boileau, T. W. M.; Clinton, S. K., Lycopene, tomato products, and prostate cancer prevention. Have we established causality? *Pure and Applied Chemistry* **2002,** 74, (8), 1435-1441.

63.   Mannisto, S.; Yaun, S. S.; Hunter, D. J.; Spiegelman, D.; Adami, H. O.; Albanes, D.; van den Brandt, P. A.; Buring, J. E.; Cerhan, J. R.; Colditz, G. A.; Freudenheim, J. L.; Fuchs, C. S.; Giovannucci, E.; Goldbohm, R. A.; Harnack, L.; Leitzmann, M.; McCullough, M. L.; Miller, A. B.; Rohan, T. E.; Schatzkin, A.; Virtamo, J.; Willett, W. C.; Wolk, A.; Zhang, S. M.; Smith-Warner, S. A., Dietary carotenoids and risk of colorectal cancer in a pooled analysis of 11 cohort studies. *American Journal of Epidemiology* **2007,** 165, (3), 246-255.

64.   Panfili, G.; Fratianni, A.; Irano, M., Normal phase high-performance liquid chromatography method for the determination of tocopherols and tocotrienols in cereals. *J Agric Food Chem* **2003,** 51, (14), 3940-4.

65.   Hakkarainen, R. V. J.; Tyopponen, J. T.; Bengtsson, S. G., Changes in the content and composition of vitamin-E in damp barley stored in airtight bins *Journal of the Science of Food and Agriculture* **1983,** 34, 1029-1038.

66.   Grela, E. R., Nutrient composition and content of antinutritional factors in spelt (Triticum spelta L) cultivars. *Journal of the Science of Food and Agriculture* **1996,** 71, 399-404.

67.   Konger, R. L., A new wrinkle on topical vitamin E and photo-inflammation: Mechanistic studies of a hydrophilic gamma-tocopherol derivative compared with alpha-tocopherol. *J Invest Dermatol* **2006,** 126, (7), 1447-9.

68.   Phoenix, J.; Edwards, R. H.; Jackson, M. J., Inhibition of Ca2+-induced cytosolic enzyme efflux from skeletal muscle by vitamin E and related compounds. *Biochem J* **1989,** 257, (1), 207-13.

69.   Shiau, C. W.; Huang, J. W.; Wang, D. S.; Weng, J. R.; Yang, C. C.; Lin, C. H.; Li, C.; Chen, C. S., alpha-Tocopheryl succinate induces apoptosis in prostate cancer

cells in part through inhibition of Bcl-xL/Bcl-2 function. *J Biol Chem* **2006,** 281, (17), 11819-25.

70.	Liu, S. M.; Lee, I. M.; Song, Y. Q.; Van Denburgh, M.; Cook, N. R.; Manson, J. E.; Buring, J. E., Vitamin E and risk of type 2 diabetes in the Women's Health Study randomized controlled trial. *Diabetes* **2006,** 55, (10), 2856-2862.

71.	Xu, Z.; Godber, J. S., Purification and identification of components of gamma-oryzanol in rice bran Oil. *J Agric Food Chem* **1999,** 47, (7), 2724-8.

72.	Hakala, P.; Lampi, A. M.; Ollilainen, V.; Werner, U.; Murkovic, M.; Wahala, K.; Karkola, S.; Piironen, V., Steryl phenolic acid esters in cereals and their milling fractions. *J Agric Food Chem* **2002,** 50, (19), 5300-7.

73.	Nystrom, L.; Makinen, M.; Lampi, A. M.; Piironen, V., Antioxidant activity of steryl ferulate extracts from rye and wheat bran. *J Agric Food Chem* **2005,** 53, (7), 2503-10.

74.	Xu, Z.; Hua, N.; Godber, J. S., Antioxidant activity of tocopherols, tocotrienols, and gamma-oryzanol components from rice bran against cholesterol oxidation accelerated by 2,2'-azobis(2-methylpropionamidine) dihydrochloride. *J Agric Food Chem* **2001,** 49, (4), 2077-81.

75.	Kikuzaki, H.; Hisamoto, M.; Hirose, K.; Akiyama, K.; Taniguchi, H., Antioxidant properties of ferulic acid and its related compounds. *J Agric Food Chem* **2002,** 50, (7), 2161-8.

76.	Yagi, K.; Ohishi, N., Action of ferulic acid and its derivatives as antioxidants. *J Nutr Sci Vitaminol (Tokyo)* **1979,** 25, (2), 127-30.

77.	Trautwein, E. A.; Schulz, C.; Rieckhoff, D.; Kunath-Rau, A.; Erbersdobler, H. F.; de Groot, W. A.; Meijer, G. W., Effect of esterified 4-desmethylsterols and -stanols or 4,4'-dimethylsterols on cholesterol and bile acid metabolism in hamsters. *Br J Nutr* **2002,** 87, (3), 227-37.

78.	Shinomiya, M.; Morisaki, N.; Matsuoka, N.; Izumi, S.; Saito, Y.; Kumagai, A.; Mitani, K.; Morita, S., Effects of gamma-oryzanol on lipid metabolism in rats fed high-cholesterol diet. *Tohoku J Exp Med* **1983,** 141, (2), 191-7.

79.	Sasaki, J.; Takada, Y.; Handa, K.; Kusuda, M.; Tanabe, Y.; Matsunaga, A.; Arakawa, K., Effects of gamma-oryzanol on serum lipids and apolipoproteins in dyslipidemic schizophrenics receiving major tranquilizers. *Clin Ther* **1990,** 12, (3), 263-8.

80.	Yoshino, G.; Kazumi, T.; Amano, M.; Tateiwa, M.; Yamasaki, T.; Takashima, S.; Iwai, M.; Hatanaka, H.; Baba, S., EFFECTS OF GAMMA-ORYZANOL ON

HYPERLIPIDEMIC SUBJECTS. *Current Therapeutic Research-Clinical and Experimental* **1989,** 45, (4), 543-552.

81.   Berger, A.; Rein, D.; Schafer, A.; Monnard, I.; Gremaud, G.; Lambelet, P.; Bertoli, C., Similar cholesterol-lowering properties of rice bran oil, with varied gamma-oryzanol, in mildly hypercholesterolemic men*. *Eur J Nutr* **2005,** 44, (3), 163-73.

82.   AACC *The Definition of Dietary Fiber* AACC: St. Paul, MN, 2001.

83.   Park, Y.; Hunter, D. J.; Spiegelman, D.; Bergkvist, L.; Berrino, F.; van den Brandt, P. A.; Buring, J. E.; Colditz, G. A.; Freudenheim, J. L.; Fuchs, C. S.; Giovannucci, E.; Goldbohm, R. A.; Graham, S.; Harnack, L.; Hartman, A. M.; Jacobs, D. R., Jr.; Kato, I.; Krogh, V.; Leitzmann, M. F.; McCullough, M. L.; Miller, A. B.; Pietinen, P.; Rohan, T. E.; Schatzkin, A.; Willett, W. C.; Wolk, A.; Zeleniuch-Jacquotte, A.; Zhang, S. M.; Smith-Warner, S. A., Dietary fiber intake and risk of colorectal cancer: a pooled analysis of prospective cohort studies. *Jama* **2005,** 294, (22), 2849-57.

84.   Southgate, D. A. T., *Dietary Fibre Analysis*. The Royal Society of Chemistry 1995.

85.   Vansoest, P. J., USE OF DETERGENTS IN ANALYSIS OF FIBROUS FEEDS .3. STUDY OF EFFECTS OF HEATING AND DRYING ON YIELD OF FIBER AND LIGNIN IN FORAGES. *Journal of the Association of Official Agricultural Chemists* **1965,** 48, (4), 785-&.

86.   Vansoest, P. J.; Wine, R. H., USE OF DETERGENTS IN ANALYSIS OF FIBROUS FEEDS .4. DETERMINATION OF PLANT CELL-WALL CONSTITUENTS. *Journal of the Association of Official Analytical Chemists* **1967,** 50, (1), 50-&.

87.   Sun, X. F.; Sun, R.; Fowler, P.; Baird, M. S., Extraction and characterization of original lignin and hemicelluloses from wheat straw. *J Agric Food Chem* **2005,** 53, (4), 860-70.

88.   Zekovic, D. B.; Kwiatkowski, S.; Vrvic, M. M.; Jakovljevic, D.; Moran, C. A., Natural and modified (1-->3)-beta-D-glucans in health promotion and disease alleviation. *Crit Rev Biotechnol* **2005,** 25, (4), 205-30.

89.   Wood, P. J., EVALUATION OF OAT BRAN AS A SOLUBLE FIBER SOURCE - CHARACTERIZATION OF OAT BETA-GLUCAN AND ITS EFFECTS ON GLYCEMIC RESPONSE. *Carbohydrate Polymers* **1994,** 25, (4), 331-336.

90.   Wood, P. J.; Anderson, J. W.; Braaten, J. T.; Cave, N. A.; Scott, F. W.; Vachon, C., PHYSIOLOGICAL-EFFECTS OF BETA-D-GLUCAN RICH FRACTIONS FROM OATS. *Cereal Foods World* **1989,** 34, (10), 878-882.

91.   Braaten, J. T.; Wood, P. J.; Scott, F. W.; Wolynetz, M. S.; Lowe, M. K.; Bradley-White, P.; Collins, M. W., Oat beta-glucan reduces blood cholesterol concentration in hypercholesterolemic subjects. *Eur J Clin Nutr* **1994,** 48, (7), 465-74.

92.   FDA *FDA allows whole oat foods to make health claim on reducing the risk of heart disease* 1997.

93.   Mackay, S.; Ball, M. J., Do beans and oat bran add to the effectiveness of a low-fat diet? *Eur J Clin Nutr* **1992,** 46, (9), 641-8.

CHAPTER 2

PHYTOCHEMICAL CONTENT AND ANTIOXIDANT ACTIVITY OF SIX DIVERSE VARIETIES OF WHOLE WHEAT[2]

Neal Okarter, Chang-Shu Liu, Mark E. Sorrells, and Rui Hai Liu

Abstract

The phytochemical content and antioxidant activity of six diverse varieties of whole wheat are reported. The free phenolic content ranged from 255 (KanQueen) to 499 (Roane) μmol gallic acid equivalents/100 g DW. The bound phenolic content ranged from 582 (Roane) to 662 (Cham1) μmol gallic acid equivalents/100 g DW. The bound fraction contributed 53.8 to 69.7% of the total phenolic content of the wheat varieties analyzed. Ferulic acid was the predominant phenolic acid found in whole wheat. Total ferulic acid content ranged from 310.8 (Caledonia) to 496.1 (KanQueen) μmol ferulic acid/100 g DW. The percentage of ferulic acid found in the insoluble-bound fraction ranged from 87.4% (Caledonia) to 97.2 % (KanQueen). Other phenolic acids, *p*-coumaric acid, syringic acid, vanillic acid, and caffeic acid were also detected. Lutein was the predominant carotenoid found in the whole wheat varieties analyzed. Zeaxanthin, β-carotene, and β-cryptoxanthin were also detected. Mainly α- and β-tocopherols and tocotrienols were found in all varieties of whole wheat though γ-tocopherol was detected in all but two varieties. β-Tocotrienol was the predominant form of vitamin E found in all varieties of whole wheat. The antioxidant activity was assessed using the oxygen radical absorbance capacity (ORAC) assay. The ORAC of the free fraction ranged from 1958 to 3749 μmol Trolox equivalents/100 g DW. The

---

ORAC of the bound fraction ranged from 3190 to 5945 μmol Trolox equivalents/100 g DW. Total phenolic content was correlated with oxygen radical absorbance capacity ($R^2 = 0.810$; $p < 0.001$). The phytochemicals found in whole grains may be responsible for the health benefit of whole grain consumption.

2.1. Introduction

Whole grain consumption has been consistently associated with reduced risk of developing chronic diseases, including cardiovascular disease (*1, 2*), type II diabetes (*3*), obesity (*4*), and cancer (*5, 6*). For this reason, the Dietary Guidelines for Americans recommend consumption of at least three-ounce equivalents of whole grains per day and to make sure at least half of total grain product consumption comes from whole grain products (*7*).

Phytochemicals are the bioactive, non-nutrient, naturally occurring plant compounds found in fruits, vegetables, and whole grains (*8*). The health benefit of whole grain consumption may be due to the unique phytochemicals found in whole grains (*9*). Whole grain phytochemicals include phenolics, compounds containing one or more aromatic rings and one or more hydroxyl groups, carotenoids, and vitamin E, amongst others. Previously, the phenolic content of whole grains had been underestimated, as most research only determined the free phenolic content and not the content of phenolics that were bound to cell wall materials (*10*). Bound phytochemicals cannot be digested by human enzymes, could survive stomach and small intestine digestion, and therefore may possibly reach the colon. The colonic microflora may release the bound phytochemicals through fermentation, and thus provide site-specific health benefits in colon or other tissues after absorption (*9*).

Whole grain phytochemicals have antioxidant activity, the ability to scavenge free radicals that may oxidize biologically relevant molecules (*9*). The antioxidant activity of whole grains has been determined using many different antioxidant activity assays (*11-17*). The oxygen radical absorbance capacity (ORAC) assay measures a sample's ability to prevent the oxidation of fluoroscein by a peroxyl radical induced by ABAP compared to the ability of various concentrations of Trolox (*18*) and has been proposed as a standard antioxidant activity assay to measure the antioxidant activity of foods (*19*).

Wheat is an important crop for the US economy. In 2007-2008, more than 60 million acres of wheat were planted yielding a projected 2 billion bushels of wheat in the US (*20*). Several different food products are made from different varieties of wheat. Hard wheat is generally used in the production of breads and cakes. Soft wheat is generally used in the production of cookies, crackers, and breakfast foods. Durum wheat, which tends to have a harder kernel than other types of wheat, is usually used in the production of pastas. The differences in hardness of the kernel, and subsequently the type of products produced from them, is due to their gluten content (*21*).

Previous studies have reported the phytochemical content of whole wheat. Adom *et al* (2003) reported the phytochemical content and antioxidant activity of 11 diverse varieties of wheat. However, the content and distribution of vitamin E, a group of lipophilic antioxidants associated with health benefit and common in grains, was not determined. Further, the antioxidant activity of the whole wheat samples was determined using the total oxyradical scavenging capacity (TOSC) assay. Moore *et al* (2005) reported the carotenoid, tocopherol, phenolic acid, and antioxidant activity of eight Maryland-grown soft wheat samples. However, the contributions from the free and bound fractions to the total phenolic content and antioxidant activity and

52

tocotrienols were not reported. Further, only soft wheat samples were used in this study. The phytochemical content and antioxidant activity of other varieties of wheat were not determined. Mpofu *et al* (2006) reported the phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. However, no distinction was made between the free and bound fractions of whole wheat. Pellegrini *et al* reported the antioxidant activity of whole wheat (*17*). Though the authors did make note the use of durum wheat, no mention was made of the variety of wheat used in the study. Further, the ferric ion reducing antioxidant power (FRAP) assay, total peroxyl radical-trapping antioxidant parameter (TRAP) assay, and trolox equivalent antioxidant capacity (TEAC) assays were used to determine the free and bound antioxidant activity of the wheat samples (*22-24*). Liyana-Pathirana and Shahidi (2006) reported the total phenolic content and antioxidant activity of hard and soft wheat. Though the authors did use the ORAC assay to distinguish between the free and bound antioxidant activity of whole wheat, a diverse range of wheat varieties was not used in the study.

The content of whole grain phytochemicals needs closer examination due to their potential health benefit in the prevention of chronic diseases. A more complete analysis of the phytochemical content and antioxidant activity of a range of diverse whole wheat samples is needed. Emphasis on the distribution of free and bound phenolic content and antioxidant activity is key to understanding the potential health benefit of whole grain consumption. The distinction between free and bound antioxidant activity needs to be made in order to understand the potential benefit of whole grain consumption. The objective of this study was to determine the phytochemical profiles and antioxidant activity of six diverse varieties of wheat.

2.2. Materials and Methods

2.2.1 Chemicals and Reagents

Sodium hydroxide, sodium sulphate, and hexanes were purchased from Fisher Scientific (Pittsburgh, PA). Acetone, sodium chloride, sodium carbonate, ethanol, ethyl acetate, and trifluroacetic acid were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Folin-Ciocalteu reagent, ferulic acid, *p*-coumaric acid, caffeic acid, *p*-hydroxybenzoic acid, carotene (α:β, 1:2) , vanilic acid, syringic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), pyrogallol, and lutein were purchased from Sigma (St. Louis, MO). Gallic acid was purchased from ICN Biomedicals, Inc. (Aurora, OH). The compound 2,2'-azobis (2-amidinopropane) dihydrochloride (ABAP) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Zeaxanthin and β-cryptoxanthin were purchased from Indofine Chemical Company Inc. (Hillsborough, NJ). All tocopherols and tocotrienols were purchased from Calbiochem (Darmstadt, Germany).

2.2.2 Grain Samples and Sample Preparation

Wheat varieties (**Table 2.1**) were provided by Dr. Mark E. Sorrells of the Cornell Small Grains Breeding and Genetics Program in the Department of Plant Breeding and Genetics at Cornell University (Ithaca, NY). All wheat varieties were milled to a fine powder using a 20, 40, and 60 mesh size screen successively and mixed thoroughly. All samples were stored at -20°C until analysis. The moisture content of all samples was determined by taking the weight of the samples before and after drying at 105 °C for 16 hours and used to express phytochemical contents to a dry weight basis (*25*).

Table 2.1. Description of the six wheat varieties used in this study.

Variety	Description	References
Stoa	Hard Red Spring	North Dakota AES, 1984 (*26*)
Roane	Soft Red Winter	Griffey *et al*, 2001 (*27*)
Caledonia	Soft White Winter	Sorrells and Cox, 2003 (*28*)
Foster	Soft Red Winter	Van Sanford *et al*, 1997 (*29*)
Cham1	White Spring Durum	Nachit *et al*, 2001 (*30*)
KanQueen	Semi-Hard Red Winter	Bayles and Clark, 1954 (*31*)

2.2.3 Extraction of Free Phenolics

Free phenolics of wheat samples were extracted using a modification of the method previously reported by Adom *et al* (2002 and 2003). Briefly, one gram of whole-wheat flour was blended with 50 mL of 80% chilled acetone. The mixture was then centrifuged at 2500 g for 10 minutes. The supernatant was removed and the remaining pellet was again extracted with 50 mL of 80% chilled acetone. The supernatants were pooled and evaporated at 45ºC to dryness. The solution was then reconstituted in methanol/hydrochloric acid (1 M; 85:15 v/v), filtered through a 0.45 μm filter, and stored at -40ºC until analysis within three days.

2.2.4 Extraction of Bound Phenolics

Bound phenolics of wheat samples were extracted using a modification of the method previously reported by our laboratory (*10, 12*). Briefly, insoluble-bound phenolics were extracted from the residue from the free phenolic extraction. The residue was first digested with 2 M sodium hydroxide at room temperature for 1 h whilst shaking under nitrogen. The mixture was then neutralized with concentrated hydrochloric acid. Hexanes were used to extract lipids in the mixture. The remaining mixture was then extracted five times with ethyl acetate. The ethyl acetate fractions were pooled and evaporated to dryness. The phenolics were reconstituted in methanol/hydrochloric acid (1 M; 85:15 v/v), filtered through a 0.45 μm filter, and stored at -40ºC until analysis within three days.

2.2.5 Extraction of Soluble-Conjugated Phenolic Compounds

Soluble-conjugated phenolic compounds were extracted from free phenolic extracts using the method routinely used in our laboratory (*10*). Free phenolics extracts were digested with 2 M sodium hydroxide for 1 h at room temperature under

nitrogen gas, and then neutralized with concentrated hydrochloric acid. The mixture was then extracted five times with ethyl acetate. The ethyl acetate fractions were pooled and evaporated to dryness under nitrogen at 35°C. The remaining residue was dissolved in methanol/hydrochloric acid (1 M; 85:15 v/v), filtered through a 0.45 μm filter, and stored at -40 °C until analysis within three days.

2.2.6 Determination of Total Phenolic Content

Total phenolic content of each wheat sample was determined using the colorimetric method described by Singleton, Orthofer, and Lamuela-Raventos (1999) and modified in our laboratory (*32, 33*). Briefly, extracts were reacted with Folin-Ciocalteu reagent and then neutralized with sodium carbonate. After 90 minutes, the absorbance of the resulting solution was measured at 760 nm. Gallic acid was used as the standard and total phenolic content was expressed as μmol of gallic acid equivalents/100 g sample DW.

2.2.7 Determination of Phenolic Acid Composition

The determination of the phenolic composition was done using an rp-HPLC-DAD method. Briefly, the mobile phase [water to pH 2.8 with acetic acid (A) and acetonitrile/water 70:30 v/v to pH 2.8 with acetic acid (B)] was delivered using a Waters 600E quaternary pump at a flow rate of 1.5 mL/min using the following gradient program: linear gradient from 0/100% – 10/90% B/A for 2.5 min, linear gradient from 10/90% – 12/88% B/A for 3.5 min, linear gradient from 12/88% – 23/77% B/A for 10 min, linear gradient from 23/77% – 95/5% B/A for 4 min, and linear gradient from 95/5% – 0/100% B/A for 6 min. The total run time was 20 min with a 6 min delay between injections. Seventy five microlit of sample were injected using a Water 717 autosampler. Separation of phenolic compounds was done using a

C18 column (5 μm, 250 mm x 4.6 mm; Grace Vydac, Baltimore, MD). Phenolic compounds were detected using a Waters 996 Photodiode Array Detector. Each injection was monitored at 282 nm. Identification of each peak was confirmed using the retention time and absorbance spectrum of each pure compound. Percent recoveries were determined by spiking a known amount of pure compound into a sample and performing the same extraction and analytical procedures. The percent recovery for ferulic acid, *p*-coumaric acid, vanillic acid, syringic acid, caffeic acid, and *p*-hydroxybenzoic acid were 101.6 ± 9.9, 99.3 ± 7.8, 83.0 ± 7.3, 85.0 ± 3.7, 89.3 ± 8.1, and 93.0 ± 7.4, respectively. The detection limits of all phenolic acids were less than 0.3 μg per injection. Data signals were acquired and processed using Waters Empower software (2002) (Waters Corp., Milford, MA).

2.2.8 Extraction of Carotenoids.

Carotenoids were extracted using the method described by Hentschel, Kranl, Hollmann, Lindhauer, Bohm, and Bitsch (2002) and modified in our laboratory (*34*). The extraction was performed under dim lighting and all sample tubes were wrapped in lightproof paper to protect carotenoids from light-induced degradation. Samples and 60 mg magnesium carbonate were placed in a 10 x 120 mm screw cap test tube to which 3 mL methanol/tetrahydrofuran (1:1, v/v) were added. The sample was vortexed, placed in a 75 ºC water bath for 5 min, vortexed again and immediately centrifuged at 2000 g for 5 minutes. After the upper solvent layer was removed to a second test tube, three additional washes of 3 mL methanol/tetrahydrofuran (1:1 v/v) were performed for complete extraction of carotenoids. Upper solvent extraction layers were pooled and vortexed with 1.5 g sodium sulphate. The solvent layer was removed and the residual rinsed at least twice with 2 mL hexane. The combined hexane solvent was evaporated to dryness under a gentle stream of nitrogen. The dry

residue was re-dissolved with 0.5 mL methanol/tetrahydrofuran (1:1, v/v) and stored under nitrogen at -20 ºC until HPLC analysis within two days.

2.2.9 Determination of Carotenoid Content.

Carotenoid content was determined using the method described by Hentschel *et al* (2002) modified by our laboratory (*34*). Briefly, the carotenoid content of each sample was determined using an rp-HPLC procedure employing a 250×4.6 mm YMC C30 column, 3 μm particle size (YMC, Waters Inc., Wilmington, NC). The mobile phase [methanol/water (95:5, v/v, A) and methyl tert-butyl ether (B)] was delivered at 1.9 mL/minute using a Water 515 HPLC pump (Water Corp., Milford, MA). The gradient program was as follows: 25/75% B/A for 11 min, 25/75% – 30/70% B/A for 2 min, 30/70% B/A for 9 min, 30/70% – 25/75% B/A for 1 min and then 25/75% B/A for at least 5 min where the next injection would occur. A Waters 2487 dual wavelength absorbance detector (Waters Corps, Milford, MA) was used for UV detection of analytes at 450 nm. Data signals were acquired and processed on a PC running the Waters Millennium software, version 3.2 (1999) (Waters Corp, Milford, MA). Percent recoveries for all carotenoids were greater than 94% (*34*). The carotenoid content of each sample extract was extrapolated from a pure carotenoid standard curve. All samples were injected via a 20 μL loop and peak heights were used for all calculations. Data were expressed as μg/100 g DW.

2.2.10 Extraction of Vitamin E.

Extraction of tocopherols and tocotrienols from whole wheat was done using a modification of the method described by Panfili, Fratianni, and Irano (2003). Briefly, 2 mL 95% ethanol, 1 mL 300 mM sodium chloride, 4 mL 500 mM pyrogallol in ethanol, 1 mL 1 M ascorbic acid, then 2 mL 10.7 M potassium hydroxide were added

to 1 g sample in a glass screw-cap tube. The headspace was flushed with nitrogen and the sample was digested for 45 min at 75°C in a constantly agitated water bath. After 45 min, the tubes were cooled to room temperature in an ice bath. After addition of 750 μL 3 M sodium chloride and 8 mL hexane/ethyl acetate (9:1, v/v), the solution was then sonicated for 10 min, and the vitamin E extracted for 5 minutes. The tube was then centrifuged at 100 g for 1 minute and the organic layer was removed. The extraction procedure was repeated twice and the organic layers from each extraction were pooled. The pooled organic layers were transferred to a separatory funnel and washed twice with 10 mL distilled water. After transfer to an Erlenmeyer flask, the solution was dehydrated with 5 g sodium sulphate. The solution was then filtered using glass wool and evaporated to dryness under a gentle stream of nitrogen at 35 °C. The residue was dissolved in 2 mL hexane/ethyl acetate/acetic acid (99.0:0.5:0.5, v/v/v), capped under nitrogen and stored at -20°C until HPLC analysis within seven days.

2.2.11 Determination of Vitamin E Content.

Chromatographic separation of tocopherols and tocotrienols was done using a np-HPLC with a fluorescence detector. Briefly, the mobile phase, hexanes/ethyl acetate/acetic acid (99.0:0.5:0.5, v/v/v) was filtered using a 0.45 μm filter and delivered by a Waters 501 Solvent Delivery System at a flow rate of 1 mL/min. One hundred microliters of sample were injected into the system and a silica column (Grace Vydac 250 x 4.6 mm, 5 μm particle size; Grace Vydac Carlsbad, CA) was used to separate the vitamers. Detection of tocopherols and tocotrienols was conducted using fluorescence detection with a Waters 474 Scanning Fluorescent Detector at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. Data signals were acquired and processed on a PC running the Waters Millennium Software,

version 3.2 (1999) (Waters Corp, Miford, MA). The detection limits for α-tocopherol and α-tocotrienol were 0.1 ng per injection. The percent recovery for α-tocopherol and α-tocotrienol were 95.2 ± 5.3 and 99.9 ± 9.9, respectively. The detection limits and percent recoveries for all other vitamers were assumed to be on the same order. Total vitamin E content was determined to be the sum of all eight vitamers and expressed as μg/g DW.

2.2.12 Antioxidant activity

The antioxidant activity was determined using oxygen radical absorbance capacity (ORAC) assay described by Huang, Ou, Hampsch-Woodill, Flanagan, and Prior (2002) and modified in our laboratory (*35, 36*). Phenolic extract dilutions were prepared with 75 mM phosphate buffer (pH 7.4). The assay was performed in black-walled 96-well plates (Corning Scientific, Corning, NY). The outside wells of the plate were not used as there was much more variation from them than from the inner wells. Each well contained 20 μL extracts or 20 μL Trolox standard (range 6.25 – 50 μM), and 200 μL fluoroscein (final concentration 0.96 μM), which were incubated at 37 ˚C for 20 minutes. After incubation, 20 μL of 119 mM ABAP was added to each well. Fluorescence intensity was measured using Fluoroskan Ascent FL plate-reader (Thermo Labsystems, Franklin, MA) at excitation of 485 nm and emission of 520 nm for 35 cycles every 5 min. ORAC was expressed as μmol Trolox equivalents/100 g DW.

2.2.13 Statistical Analysis.

Data were reported as mean ± standard deviation for triplicate determinations of each sample. ANOVA and Tukey's comparison test were performed using Minitab

Statistical Software version 15 (State College, PA) to identify differences between values. Statistical significance was defined to be at a level of $p < 0.05$.

2.3 Results

2.3.1 Total Phenolic Content.

The free phenolic content ranged from 254.7 (KanQueen) to 499.5 (Roane) µmol gallic acid equivalents/100 g DW. The ratio of bound phenolics to free phenolic ranged from 1.2 (Roane) to 2.3 (KanQueen). The bound phenolic content ranged from 581.9 (Roane) to 662.4 (Cham1) µmol gallic acid equivalents/100 g DW. The total phenolic content ranged from 841 (KanQueen) to 1099 (Stoa) µmol gallic acid equivalents/100 g DW (**Table 2.2**). The total phenolic content of Stoa, Roane, and Cham1 were significantly different ($p < 0.05$) from the total phenolic content of Caledonia, Foster, and KanQueen.

2.3.2 Phenolic Acid Composition.

Ferulic acid was the predominant phenolic acid found in each variety of whole wheat and was found in the free, soluble-conjugated, and insoluble bound fractions (**Table 2.3**). Free ferulic acid content ranged from 4.3 (Foster) to 20.1 (Roane) µmol ferulic acid/100 g DW. Soluble-conjugated ferulic acid ranged from 9.6 (KanQueen) to 30.5 (Caledonia) µmol ferulic acid/100 g DW. The soluble-conjugated ferulic acid content of Caledonia was significantly different ($p < 0.05$) from all other soluble-conjugated ferulic acid contents. The bound ferulic acid content ranged from 271.5 (Caledonia) to 482.1 (KanQueen) µmol ferulic acid/100 g DW. The bound ferulic acid content of KanQueen was significantly different ($p < 0.05$) from all other bound ferulic acid contents. Total ferulic acid content ranged from 310.8 (Caledonia) to

Table 2.2 – Total phenolic content of wheat varieties. Values expressed as μmol gallic acid equivalents/100 g DW (mean ± standard deviation, n = 3). Percent contribution to total phenolic content in parentheses. Values with no letter is common are significantly different (p < 0.05).

	Free	Bound	Total
KanQueen	245.7 ± 10.0 g (30.3)	586.2 ± 53.9 cd (69.7)	841 ± 62.3 b
Caledonia	307.0 ± 21.0 fg (35.9)	548.8 ± 1.8 d (64.1)	856 ± 19.5 b
Foster	332.5 ± 24.8 fg (35.7)	598.5 ± 24.6 cd (64.3)	931 ± 40.7 b
Cham1	398.6 ± 8.5 ef (37.6)	662.4 ± 35.4 c (62.4)	1061 ± 36.2 a
Roane	499.5 ± 27.0 de (46.2)	581.9 ± 49.0 cd (53.8)	1081 ± 52.2 a
Stoa	459.1 ± 18.5 de (41.8)	639.7 ± 34.9 cd (58.2)	1099 ± 19.1 a

496.1 (KanQueen) μmol ferulic acid/100 g DW. The percentage of ferulic acid found in the insoluble-bound fraction ranged from 87.4 (Caledonia) to 97.2 % (KanQueen).

p-Coumaric acid was found in the free, soluble-conjugated, and insoluble bound fractions. Free *p*-coumaric acid content ranged from 9.2 (Foster) to 25.4 (Caledonia) μmol *p*-coumaric acid/100 g DW. The free *p*-coumaric acid of Caledonia was significantly different ($p < 0.05$) from all other free *p*-coumaric acid contents. Soluble-conjugated *p*-coumaric acid ranged from 7.4 (Stoa) to 10.0 (Caledonia) μmol *p*-coumaric acid/100 g DW. Insoluble bound *p*-coumaric acid content ranged from 15.9 (Cham1) to 29.0 (KanQueen) μmol *p*-coumaric acid/100 g DW. The bound *p*-coumaric acid content of KanQueen was significantly different ($p < 0.05$) from all other bound *p*-coumaric acid contents. The percentage of *p*-coumaric acid found in the insoluble bound fraction ranged 32.3 (Caledonia) to 63.4% (KanQueen).

Syringic acid was found in the soluble-conjugated and insoluble-bound fractions. Soluble-conjugated syringic acid content ranged from 4.9 (Cham1) to 11.5 (Caledonia) μmol syringic acid/100 g DW. Insoluble-bound syringic acid content ranged from 3.1 (Caledonia) to 9.8 (KanQueen) μmol syringic acid/100 g DW. The bound syringic acid content of KanQueen was significantly different ($p < 0.05$) from all other bound syringic acid contents. No insoluble-bound syringic acid was detected in Foster. The percentage of syringic acid in the insoluble bound fraction ranged from 21.2 (Caledonia) to 63.2% (KanQueen).

Vanillic acid was found only in the soluble-conjugated fraction. Soluble conjugated vanillic acid contents ranged from 4.3 (KanQueen) to 8.3 (Caledonia) μmol vanillic acid /100 g DW.

Caffeic acid was only found in the insoluble bound fraction. Caffeic acid contents ranged from 3.2 (Caledonia) to 7.4 (KanQueen) μmol caffeic acid/100 g DW.

Table 2.3 – Phenolic acid composition of six diverse varieties of wheat. Values expressed as µmol phenolic acid/100 g DW (mean ± standard deviation, n = 3). Percent contribution to total phenolic acid content is in parentheses. Values with no letters in common within each column are significantly different (p < 0.05). nd – not detected

	Free	Soluble Conjugated	Insoluble-Bound	Total
		Ferulic Acid		
Caledonia	8.7 ± 0.7 b (2.8%)	30.5 ± 2.7 a (9.8%)	271.5 ± 5.1 b (87.4%)	310.8 ± 7.2 b
Cham1	11.6 ± 0.7 b (3.3%)	11.8 ± 1.1 b (3.4%)	326.7 ± 29.8 b (93.3%)	350.1 ± 28.9 b
Foster	4.3 ± 1.4 c (1.3%)	12.3 ± 2.1 b (3.8%)	307.7 ± 14.2 b (94.9%)	324.2 ± 15.6 b
KanQueen	4.4 ± 0.6 c (0.9%)	9.6 ± 1.0 b (1.9%)	482 1 ± 90.3 a (97.2%)	496.1 ± 90.2 a
Roane	20.1 ± 0.5 a (6.2%)	11.4 ± 0.9 b (3.6%)	290.4 ± 36.3 b (90.2%)	321.8 ± 36.4 b
Stoa	10.2 ± 1.3 b (2.7%)	11.5 ± 0.7 b (3.0%)	361.8 ± 19.6 b (94.3%)	383.5 ± 21.2 b
		p-Coumaric Acid		
Caledonia	25.4 ± 4.3 a (48.6%)	10.0 ± 0.6 a (19.1%)	16.9 ± 0.5 b (32.3%)	52.3 ± 4.0 a
Cham1	10.2 ± 0.6 b (30.4%)	7.5 ± 0.2 bc (22.3%)	15.9 ± 1.3 b (47.3%)	33.5 ± 0.5 c
Foster	9.2 ± 1.8 b (26.0%)	9.2 ± 1.3 ab (26.0%)	17.0 ± 1.5 b (48.0%)	35.5 ± 4.4 c
KanQueen	9.5 ± 1.2 b (20.4%)	8.0 ± 0.4 b (17.2%)	29.0 ± 2.4 a (62.4%)	46.5 ± 1.5 ab
Roane	14.4 ± 1.1 b (35.0%)	7.7 ± 0.2 b (18.7%)	19.0 ± 1.5 b (46.3%)	41.1 ± 2.3 bc
Stoa	11.2 ± 1.7 b (32.4%)	7.4 ± 0.1 c (21.4%)	16.0 ± 1.6 b (46.2%)	34.7 ± 1.2 c

Table 2.3 (continued)

	Free	Soluble-Conjugated	Insoluble-Bound	Total
		Syringic Acid		
Caledonia	nd	11.5 ± 0.3 a (78.8%)	3.1 ± 0.6 b (21.2%)	14.6 ± 0.3 a
Cham1	nd	4.9 ± 0.8 c (54.4%)	4.1 ± 0.7 b (45.6%)	9.0 ± 1.5 b
Foster	nd	8.7 ± 2.5 ab (100%)	nd	8.7 ± 2.5 b
KanQueen	nd	5.7 ± 1.5 bc (36.8%)	9.8 ± 2.3 a (63.2%)	15.5 ± 3.3 a
Roane	nd	6.3 ± 0.7 bc (60.0%)	4.2 ± 0.6 b (40.0%)	10.5 ± 1.3 ab
Stoa	nd	7.6 ± 0.1 bc (65.0%)	4.1 ± 0.6 b (35.0%)	11.7 ± 0.6 ab
		Vanillic Acid		
Caledonia	nd	8.3 ± 2.0 a (100%)	nd	8.3 ± 2.0 a
Cham1	nd	6.5 ± 0.5 ab (100%)	nd	6.5 ± 0.5 ab
Foster	nd	7.8 ± 1.6 a (100%)	nd	7.8 ± 1.6 a
KanQueen	nd	4.3 ± 1.4 b (100%)	nd	4.3 ± 1.4 b
Roane	nd	7.6 ± 0.5 a (100%)	nd	7.6 ± 0.5 a
Stoa	nd	6.0 ± 0.2 ab (100%)	nd	6.0 ± 0.2 ab
		Caffeic Acid		
Caledonia	nd	nd	4.4 ± 1.1 c (100%)	4.4 ± 1.1 c
Cham1	nd	nd	7.4 ± 0.9 bc (100%)	7.4 ± 0.9 bc
Foster	nd	nd	5.9 ± 0.5 bc (100%)	5.9 ± 0.5 bc
KanQueen	nd	nd	11.6 ± 0.4 a (100%)	11.6 ± 0.4 a
Roane	nd	nd	7.5 ± 1.0 b (100%)	7.5 ± 1.0 b
Stoa	nd	nd	8.2 ± 2.0 b (100%)	8.2 ± 2.0 b

The bound caffeic acid content of KanQueen was significantly different ($p < 0.05$) from all other bound caffeic acid contents.

2.3.3 Carotenoid Content.

Total carotenoid content ranged from 148 (KanQueen) to 271 (Foster) µg/100g DW. Lutein content was the highest among the carotenoids tested and ranged from 67.4 (KanQueen) to 211.1 (Foster) µg/100g DW. Zeaxanthin content ranged from 25.3 (Cham1) to 52.7 (Caledonia) µg/100g DW. β-Cryptoxanthin content ranged from 11.8 (Caledonia) to 19.5 (Roane) µg/100g DW. None of the β-cryptoxanthin contents were significantly different ($p > 0.05$). β-Carotene content ranged from 17.6 (Cham1) to 36.2 (Foster) µg/100g DW (**Table 2.4**). α-Carotene was not detected in any variety tested.

2.3.4 Vitamin E Content.

Total vitamin E content ranged from 13.4 (Cham1) to 19.6 (KanQueen) µg/g DW (**Table 2.5**). In all samples, β-tocotrienol was the predominant form of vitamin E. β-Tocotrienol content ranged from 8.6 (Roane) to 11.9 (Caledonia) µg/g DW. Mainly α and β-tocopherols and -tocotrienols were detected in the samples. Four of the six varieties of wheat contained γ-tocopherol. The content of γ-tocopherol was low and ranged from 0.1 (Cham1) to 0.9 (KanQueen) µg/g DW. α-Tocopherol content ranged from 0.7 (Cham1) to 5.2 (KanQueen) µg/g DW.

2.3.5 Oxygen Radical Absorbance Capacity (ORAC).

The free ORAC ranged from 1958 (KanQueen) to 3749 (Stoa) µmol Trolox equivalents/100 g DW. The bound ORAC ranged from 3190 (KanQueen) to 5945 (Roane) µmol Trolox equivalents/100 g DW. The total ORAC of the wheat samples

Table 2.4 – Carotenoid (lutein, zeaxanthin, β-cryptoxanthin, and β-carotene) content and distribution of wheat varieties (mean ± standard deviation, n = 3). Values with no letters in common within each column are significantly different (p < 0.05).

	Lutein	Zeaxanthin	β-Cryptoxanthin	β-Carotene
KanQueen	67.4 ± 21.5 c	27.8 ± 1.4 c	15.9 ± 2.2	19.7 ± 3.6 b
Stoa	97.6 ± 5.4 c	35.1 ± 1.7 b	16.0 ± 2.5	17.9 ± 4.4 b
Roane	119.0 ± 32.8 bc	44.1 ± 6.6 ab	19.5 ± 3.4	23.6 ± 1.2 b
Caledonia	173.5 ± 22.4 ab	52.7 ± 3.8 a	11.8 ± 2.2	23.1 ± 7.5 b
Cham1	207.6 ± 33.6 a	25.3 ± 2.6 c	17.4 ± 3.2	17.6 ± 3.6 b
Foster	211.1 ± 5.8 a	39.8 ± 0.1 b	14.5 ± 3.3	36.2 ± 8.2 a

Table 2.5 – Vitamin E content of wheat varieties. Values expressed as μg/g DW (mean ± standard deviation, n = 3). Values with no letters in common within each column are significantly different (p < 0.05). nd – not detected

	Tocopherols				Tocotrienols				Total VE
	α	β	γ	δ	α	β	γ	δ	
Caledonia	0.7 ± 0.1 c	1.8 ± 0.2 c	0.2 ± 0.03 c	nd	0.4 ± 0.1 b	7.2 ± 0.9 b	nd	nd	10.2 ± 1.3 b
Roane	1.2 ± 0.3 bc	2.9 ± 0.6 ab	0.2 ± 0.01 b	nd	0.5 ± 0.1 b	8.6 ± 1.2 ab	nd	nd	13.4 ± 2.1 ab
Cham1	0.7 ± 0.1 c	1.2 ± 0.3 c	0.1 ± 0.03 c	nd	1.3 ± 0.1 a	9.2 ± 1.0 ab	nd	nd	13.8 ± 2.1 ab
Stoa	1.9 ± 0.3 bc	2.1 ± 0.3 bc	nd	nd	0.6 ± 0.1 b	9.9 ± 0.9	nd	nd	14.5 ± 1.7 ab
Foster	4.2 ± 2.0 ab	1.3 ± 0.1 c	1.3 ± 0.1 a	nd	1.4 ± 0.2 a	10.0 ± 0.9 ab	nd	nd	18.1 ± 2.6 ab
KanQueen	5.2 ± 2.0 ab	1.5 ± 0.4 c	0.9 ± 0.1 b	nd	1.0 0.2 a	11.2 ± 1.3 a	nd	nd	19.8 ± 0.8 a

Table 2.6 – ORAC values of wheat varieties (mean ± standard deviation, n = 3). Percent contribution to total ORAC is in parentheses. Values with no letters in common are significantly different (p < 0.05).

	Free ORAC	Bound ORAC	Total ORAC[a]
KanQueen	1958 ± 251 g (38.0)	3190 ± 687 fg (62.0)	5148 ± 486 def
Caledonia	2703 ± 653 fg (39.4)	4159 ± 316 f (60.6)	6862 ± 342 cd
Cham1	2552 ± 394 g (35.6)	4594 ± 577 ef (64.4)	7174 ± 962 bc
Foster	3545 ± 426 fg (47.2)	3960 ± 631 f (52.8)	7505 ± 424 bc
Roane	2694 ± 192 fg (31.2)	5945 ± 754 de (68.8)	8639 ± 830 ab
Stoa	3749 ± 423 fg (39.0)	5867 ± 352 de (61.0)	9616 ± 752 a

[a]Sum of free and bound ORAC

ranged from 5148 (KanQueen) to 9616 (Stoa) µmol Trolox equivalents/100 g DW (**Table 2.6**). Total phenolic content was correlated with ORAC ($R^2 = 0.810$; $p < 0.001$).

2.4 Discussions

2.4.1 Total Phenolic Content.

Phenolics are compounds with one or more aromatic ring and one or more hydroxyl groups (*37*). Phenolic compounds from the insoluble bound fraction of whole wheat are of particular importance because of their ability to scavenge free radicals and prevent the oxidation of biologically important molecules in the colon (*10, 38*). The total phenolic contents we reported here are similar to the total phenolic content values for 11 diverse varieties of whole wheat reported earlier (*12*), and ranged from 710 to 860 µmol gallic acid equivalents/100 g FW. Moore *et al* (2005) reported the total phenolic content of eight Maryland-grown soft wheat samples, ranged from 235 to 470 µmol gallic acid equivalents/100 g. These values were significantly lower than those reported in the present study and Adom *et al* (2003). This may be due to differences in extraction procedures. Because no alkali hydrolysis and further extraction was performed, the authors only extracted phenolic compounds from the free fraction. This would lead to an underestimation of the total phenolic content. The total phenolic content values reported by Moore *et al* (2005) are similar to the free phenolic content values reported in this study.

The total phenolic content of wheat has also been reported in ferulic acid equivalents. Mpofu *et al* (2006) reported that the average total phenolic content of six wheat genotypes was 958 µmol ferulic acid equivalents/100 g. Liyana-Pathirana and Shahidi (2006) reported that the total phenolic content of soft and hard wheat was

1966 and 1780, respectively. The bound fraction contributed 56 and 62% to the total phenolic content, respectively. This finding is consistent with the percent contribution from the bound fraction in this study. On average, the bound fraction accounted for 62% of the total phenolic content.

2.4.2 Phenolic Acid Composition.

Phenolic acids are derivatives of hydroxybenzoic acid or hydroxycinnamic acid. Ferulic acid, a hydroxycinnamic acid derivative, is the predominant phenolic acid found in whole grains (*39, 40*) and whole wheat (*15, 41*). Sosulski *et al* (1982) reported that the total ferulic acid content was 32.7 µmol ferulic acid/100 g with the bound ferulic acid content accounting for 92% of the total ferulic acid content. Adom *et al* (2003) reported that the total ferulic acid content of 11 diverse varieties of whole wheat ranged from 147.7 to 303.0 µmol ferulic acid/100 g with the bound ferulic acid accounting for more than 97% of the total ferulic acid content. Moore *et al* reported that the total ferulic acid content of eight Maryland-grown soft wheat samples ranged from 234.8 to 320.0 µmol ferulic acid/100 g with the bound ferulic acid accounting for more than 89% of the total ferulic acid content (*15*). Li, Shewry, and Ward (2008) reported that the total ferulic acid content of spring wheat and winter wheat were 209.2 and 205.4 µmol ferulic acid/100 g, respectively.

Matilla *et al* reported that the total ferulic acid content of whole wheat flour was 458.3 µmol ferulic acid/100 g DW (*39*). Mpofu *et al* reported that the total ferulic acid content of hard spring wheat ranged from 191.0 to 227.1 µmol ferulic acid/100 g (*16*). Siebenhandl *et al* reported that the total ferulic acid content of a purple pericarp wheat was 438.5 µmol ferulic acid/100 g FW (*41*). The contribution from the bound fraction to the total ferulic acid content was not reported in any of these studies.

In this study, the total ferulic acid content ranged from 310.8 to 496.1 μmol ferulic acid/100 g DW. The bound fraction contributed 87 to 97% to the total ferulic acid content. These findings are similar to those previously reported (*12, 15, 16, 39, 41*).

Other phenolic acids were also found in significant quantities in whole wheat. The hydroxycinnamic acid derivatives are more prevalent than the hydroxybenzoic acid derivatives. The second most abundant phenolic acid found in whole wheat is *p*-coumaric acid. Moore *et al* (2005) reported the *p*-coumaric acid content of eight varieties of Maryland-grown soft wheat. The average total *p*-coumaric acid content was 7.4 μmol/100 g. The bound fraction accounted for nearly 90% of the total *p*-coumaric acid content. Matilla *et al* reported the total *p*-coumaric acid and total caffeic contents of whole wheat. The total *p*-coumaric acid and total caffeic acid contents of whole wheat were 22.5 and 20.5 μmol/100 g DW, respectively (*39*). Mpofu *et al* (2006) reported the total *p*-coumaric acid and total caffeic acid content of six genotypes of wheat. The average total *p*-coumaric acid and caffeic acid contents were 18.9 and 5.6 μmol/100 g, respectively. Siebenhandl *et al* (2007) reported that the total *p*-coumaric acid content of purple pericarp wheat was 14.8 μmol/100 g DW. Li *et al* (2008) reported that the total *p*-coumaric acid content of spring wheat and winter wheat was 6.7 and 9.6 μmol *p*-coumaric acid/100 g, respectively. However, the contribution of *p*-coumaric acid from the bound fraction varied between the two types of wheat. The bound fraction of winter wheat accounted for 63% of the total *p*-coumaric acid content, while spring wheat accounted for 37%. Winter wheat contained 0.2 μmol caffeic acid/100 g though no caffeic acid was found in the spring wheat (*42*). All of the caffeic acid in the winter wheat was from the free fraction.

The *p*-coumaric acid contents reported in the present study are higher than those previously reported in literature. The percent contribution to the total *p*-

coumaric acid content was most similar to that reported by Li *et al* (2008). In this study, the bound *p*-coumaric acid content from the winter wheat varieties contributed 47.3% to the total *p*-coumaric acid content compared to 63.3%, as reported by Li *et al* (2008). Caffeic acid contents reported across a number of studies appear to be more variable than ferulic acid and *p*-coumaric acid contents. In this study, the average caffeic acid content was 7.5 μmol/100 g DW, with the bound fraction contributing all of the caffeic acid found. This values agrees most with that reported by Mpofu *et al* (2006).

Hydroxybenzoic acid derivatives have also been reported in whole wheat. Moore *et al* (2005) reported that the vanillic and syringic acid contents of eight varieties of Maryland-grown soft wheat were 6.2 and 5.3 μmol/100 g. The soluble conjugated fraction contributed the most to the total contents of both vanillic acid and syringic acid, 51 and 65.6%, respectively. Only two of the eight varieties had any syringic acid in the free fraction. The bound fraction contributed 38.5 and 34.3% to the total contents of vanillic acid and syringic acid, respectively. No *p*-hydroxybenzoic acid was detected in this study. Matilla *et al* (2005) reported the content of hydroxybenzoic acid derivatives from whole wheat. The total vanillic, syringic, and *p*-hydroxybenzoic acid contents were 8.9, 6.6, and 5.4 μmol/100 g DW, respectively (*39*). Mpofu *et al* (2006) reported that the total content of vanillic and syringic acids, were 5.4 and 5.7 μmol/100 g, respectively. Siebenhandl *et al* (2007) reported that the total vanillic acid content of purple pericarp wheat was 20.9 μmol/100 g. Li *et al* (2008) reported that the total vanillic and syringic acid contents of winter wheat were 12.4 and 8.9 μmol/100 g. The soluble conjugated fraction contributed the most to the total vanillic and syringic acid contents of winter, 67.5 and 61.9%, respectively. Li *et al* (2008) also reported that the total vanillic and syringic acid contents of spring wheat were 11.6 and 8.8 μmol/100 g, respectively. The soluble

conjugated fraction contributed the most to the total vanillic and syringic acid contents of spring wheat, 71.8 and 63.6%, respectively.

The total content of hydroxybenzoic acid derivatives reported in the present study is consistent with previously reported values. The average total vanillic acid and syringic acid contents were 6.8 and 11.7 μmol/100 g DW. However, there were slight differences in percent contribution from the various fractions. In the present study, no vanillic acid was detected in the free or bound fractions. Further, no syringic acid was detected in the free fraction, which is consistent with the results reported by Moore *et al* (2005). No *p*-hydroxybenzoic acid was detected in any variety of wheat, which is also consistent with data reported by Moore *et al* (2005), but not consistent with that reported by Matilla *et al* (2005).

2.4.3 Carotenoid Content.

Hentshel *et al* (2002) reported that the carotenoid content of four durum wheat samples averaged to roughly 200 μg/100 g. In this study, no β-carotene was detected. Further, the total carotenoid content of the durum wheat sample was 240 μg/100 g. Adom *et al* (2003) reported the carotenoid content of 11 diverse varieties of whole wheat. However, the β-carotene content of each sample was not reported. The lutein, zeaxanthin, and β-cryptoxanthin values reported in this study are similar to those reported by Adom *et al* (2003), though the values are slightly higher and the range of values is narrower. Moore *et al* (2005) reported the carotenoid content of eight Maryland-grown soft wheat samples. The carotenoid values reported in that study are similar to those reported in this study. However, β-cryptoxanthin content was not reported. Here the carotenoid content of four different carotenoids is reported. Hidalgo, Brandolini, Pompei, and Piscozzi (2006) reported the carotenoid content of 54 cultivars of Einkorn wheat, 6 durum wheat cultivars, and 5 bread wheat cultivars

grown across Europe. The average total carotenoid content of the durum wheat, bread wheat, and Einkorn wheat varieties was 320, 195, and 841 μg/100 g DW, respectively.

The total carotenoid content of the six diverse varieties of wheat reported in this study ranged from 148 to 271 μg/100 g DW. However, one variety of durum wheat (Cham1) was used in the present study. The values reported for lutein and β-carotene in this study are also in agreement with those reported by Hidalgo *et al* (2006) and Adom *et al* (2006).

2.4.4 Vitamin E Content.

All varieties of wheat contained more tocotrienols than tocopherols (Table 3). The tocotrienol:tocopherol ratio ranged from 1.9 (KanQueen) to 5.3 (Cham1). Panfili *et al* (2003) reported that the average tocotrienol:tocopherol ratio of soft wheat and durum wheat was 1.9 and 3.3, respectively. Here, we report that the average tocotrienol:tocopherol ratio of soft wheat and durum wheat was 3.0 and 5.3, respectively.

Panfili *et al* reported the vitamin E content of durum and soft wheat (*43*). Total vitamin E content for durum wheat and soft wheat was 60.6 and 74.3 μg/g DW, respectively. In both cases, β-tocotrienol was the predominant form of vitamin E. As Panfili *et al* (2003) noted, there are no standardized methods for the extraction and analysis of vitamin E from cereals. Panfili *et al* showed that a methanol extraction or a solvent extraction without prior saponification resulted in lower recovery of vitamin E when compared to the saponification followed by organic solvent extraction (*43*). Moore *et al* determined the tocopherol content of eight Maryland-grown soft wheat samples using ESI-MSMS (*15*). Tocotrienol content was not reported. Only α-tocopherol was detected in these wheat samples. Not being able to detect β-tocopherol may be due to the extraction procedure, in which, methanol/THF 1:1 v/v was used

with no saponification. Hidalgo *et al* (2006) reported the vitamin E content of 54

cultivars of Einkorn wheat, 6 durum wheat cultivars, and 5 bread wheat cultivars

grown across Europe. The total vitamin E content for the durum wheat, bread wheat,

and Einkorn wheat varieties was 50.5, 61.5, and 78.0 μg/g DW, respectively. These

values are at least 2-fold higher than those reported in this study.

The vitamin E contents reported in the present study are lower than previously

reported values (*43-45*). However, the distribution of tocopherols and tocotrienols

reported in the present study is consistent with previously reported values. β-

Tocotrienol was the predominant form of vitamin E accounting for roughly half of the

total vitamin E content. γ-Tocopherol was detected in five of the six varieties

analyzed. Grela reported the presence of γ-tocopherol in durum wheat (*46*). The

present study is consistent with the data reported by Grela, finding γ-tocopherol not

only in durum wheat, but also in hard and soft red wheat. The ability to detect γ-

tocopherol in wheat may be due to the efficiencies of the extraction and analytical

methods.

2.4.5 Oxygen Radical Absorbance Capacity.

The antioxidant activity of whole wheat extracts have been determined using

previously reported antioxidant activity assays (*11, 12, 15, 17, 41*). Comparing the

antioxidant capacity of the wheat varieties used in these studies is difficult because the

different antioxidant capacity assay have not been standardized (*47*).

Moore *et al* (2005) reported the ORAC of eight Maryland-grown soft wheat

samples. ORAC values ranged from 3290 to 4770 μmol Trolox equivalents/100 g.

These values were similar to the free ORAC values reported in this study and much

lower than the total ORAC values reported. Total phenolic content was correlated

with the ORAC ($r = 0.908$; $p = 0.01$). However, Moore *et al* (2005) did not report the

ORAC contribution from the free and bound fraction. Liyana-Pathirana and Shahidi (2006) reported that the bound fraction from hard and soft winter wheat contributed 87.4% and 86.6% to the total ORAC, respectively.

In the present study, the total ORAC values of six diverse varieties of wheat ranged from 5148 to 9616 μmol Trolox equivalents/100 g DW. The free ORAC ranged from 1958 to 3749 μmol Trolox equivalents/100 g DW. The bound ORAC ranged from 3190 to 5945 μmol Trolox equivalents/100 g DW. Three of the six varieties of wheat had bound ORAC values that were significantly different ($p < 0.05$) from the free ORAC (Table 5). The bound fraction contributed 52.8 to 68.8% of the total ORAC.

2.4.6 Summary

Whole grain consumption is associated with reduced risk of chronic diseases including cardiovascular disease, type II diabetes, obesity, and cancer. Here the phytochemical content and antioxidant activity of six diverse varieties of whole wheat are reported. The bound fraction contributed 53.8 to 69.7% of the total phenolic content of the wheat varieties analyzed. Ferulic acid was the predominant phenolic acid found in whole wheat. Other phenolic acids, *p*-coumaric acid, syringic acid, vanillic acid, and caffeic acid were also detected. The carotenoid content is also reported in this study. Lutein was the predominant carotenoid found in the whole wheat varieties analyzed. Zeaxanthin, β-carotene, and β-cryptoxanthin were also detected. The vitamin E content of whole wheat is reported in the present study. Mainly α and β-tocopherols and -tocotrienols were found in all varieties of whole wheat though γ-tocopherol was detected in all but two varieties. β-Tocotrienol was the predominant form of vitamin E found in all varieties of whole wheat. The antioxidant activity was assessed using the ORAC assay. The ORAC of the free

fraction ranged from 1958 to 3749 μmol Trolox equivalents/100 g DW. The ORAC of

the bound fraction ranged from 3190 to 5945 μmol Trolox equivalents/100 g DW.

Total phenolic content was correlated with oxygen radical absorbance capacity ($R^2 =$

0.810; $p < 0.001$). The phytochemicals found in whole grains may be responsible for

the health benefit of whole grain consumption.

LITERATURE CITED

1.   Anderson, J. W., Whole grains and coronary heart disease: the whole kernel of truth. *Am J Clin Nutr* **2004,** 80, (6), 1459-60.

2.   Jacobs, D. R., Jr.; Meyer, K. A.; Kushi, L. H.; Folsom, A. R., Whole-grain intake may reduce the risk of ischemic heart disease death in postmenopausal women: the Iowa Women's Health Study. *Am J Clin Nutr* **1998,** 68, (2), 248-57.

3.   Montonen, J.; Knekt, P.; Jarvinen, R.; Aromaa, A.; Reunanen, A., Whole-grain and fiber intake and the incidence of type 2 diabetes. *Am J Clin Nutr* **2003,** 77, (3), 622-629.

4.   Liu, S.; Willett, W. C.; Manson, J. E.; Hu, F. B.; Rosner, B.; Colditz, G., Relation between changes in intakes of dietary fiber and grain products and changes in weight and development of obesity among middle-aged women. *Am J Clin Nutr* **2003,** 78, (5), 920-7.

5.   Jacobs, D. R., Jr.; Slavin, J.; Marquart, L., Whole grain intake and cancer: a review of the literature. *Nutr Cancer* **1995,** 24, (3), 221-9.

6.   Schatzkin, A.; Mouw, T.; Park, Y.; Subar, A. F.; Kipnis, V.; Hollenbeck, A.; Leitzmann, M. F.; Thompson, F. E., Dietary fiber and whole-grain consumption in relation to colorectal cancer in the NIH-AARP Diet and Health Study. *Am J Clin Nutr* **2007,** 85, (5), 1353-60.

7.   USDA *Dietary Guidelines for Americans 2005*; USDA Human Nutrition Information Service Hyattsville, MD, 2005.

8.   Liu, R. H., Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J Nutr* **2004,** 134, (12 Suppl), 3479S-3485S.

9.   Liu, R. H., Whole grain phytochemicals and health. *Journal of Cereal Science* **2007,** 46, (3), 207-219.

10.   Adom, K. K.; Liu, R. H., Antioxidant activity of grains. *J Agric Food Chem* **2002,** 50, (21), 6182-7.

11.   Adom, K. K.; Liu, R. H., Rapid peroxyl radical scavenging capacity (PSC) assay for assessing both hydrophilic and lipophilic antioxidants. *J Agric Food Chem* **2005,** 53, (17), 6572-80.

12.   Adom, K. K.; Sorrells, M. E.; Liu, R. H., Phytochemical profiles and antioxidant activity of wheat varieties. *J Agric Food Chem* **2003,** 51, (26), 7825-34.

13.   Liyana-Pathirana, C.; Shahidi, F., Antioxidant activity of wheat extracts as affected by in vitro digestion. *Biofactors* **2004,** 21, (1-4), 325-8.

14.   Liyana-Pathirana, C. M.; Shahidi, F., Antioxidant activity of commercial soft and hard wheat (Triticum aestivum L.) as affected by gastric pH conditions. *J Agric Food Chem* **2005,** 53, (7), 2433-40.

15.   Moore, J.; Hao, Z.; Zhou, K.; Luther, M.; Costa, J.; Yu, L. L., Carotenoid, tocopherol, phenolic acid, and antioxidant properties of Maryland-grown soft wheat. *J Agric Food Chem* **2005,** 53, (17), 6649-57.

16.   Mpofu, A.; Sapirstein, H. D.; Beta, T., Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. *Journal of Agricultural and Food Chemistry* **2006,** 54, (4), 1265-1270.

17.   Pellegrini, N.; Serafini, M.; Salvatore, S.; Del Rio, D.; Bianchi, M.; Brighenti, F., Total antioxidant capacity of spices, dried fruits, nuts, pulses, cereals and sweets consumed in Italy assessed by three different in vitro assays. *Mol Nutr Food Res* **2006,** 50, (11), 1030-8.

18.   Ou, B. X.; Hampsch-Woodill, M.; Prior, R. L., Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *Journal of Agricultural and Food Chemistry* **2001,** 49, (10), 4619-4626.

19.   Prior, R. L.; Wu, X.; Schaich, K., Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agric Food Chem* **2005,** 53, (10), 4290-302.

20.   Vocke, G.; Allen, E. *Wheat Situation and Outlook Yearbook* USDA Economic Research Service - Market and Trade Economics Division 2007.

21.   Distaam, E. M.; Carcea, M., Pasta from nontraditional raw materials *Cereal Foods World* **2001,** 46, 522-530.

22.   Benzie, I. F.; Szeto, Y. T., Total antioxidant capacity of teas by the ferric reducing/antioxidant power assay. *J Agric Food Chem* **1999,** 47, (2), 633-6.

23.   Ghiselli, A.; Serafini, M.; Maiani, G.; Azzini, E.; Ferroluzzi, A., A Fluorescence-Based Method for Measuring Total Plasma Antioxidant Capability. *Free Radical Biology and Medicine* **1995,** 18, (1), 29-36.

24.   Miller, N. J.; Rice-Evans, C.; Davies, M. J.; Gopinathan, V.; Milner, A., A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin Sci (Lond)* **1993,** 84, (4), 407-12.

25.   AOAC, *AOAC Official Methods of Analysis* 15 ed.; Washington DC, 1990; Vol. 2.

26.   NDAES; USDA-ARS Stoa - Hard Red Spring Wheat

27.   Griffey, C. A.; Starling, T. M.; Price, A. M.; Sisson, W. L.; Das, M. K.; Pridgen, T. H.; Vaughn, M. E.; Rohrer, W. L.; Brann, D. E., Roane wheat. *Crop Sci.* **2001,** 41, 1359.

28.   Sorrells, M. E.; Cox, W. J., Registration of Caledonia wheat *Crop Sci.* **2003**.

29.   VanSanford, D. A.; Swanson, C. S.; Pearce, W. L.; Tutt, C. R.; Tomes, L. J.; Hershman, D. E., Registration of 'Foster' wheat. *Crop Science* **1997,** 37, (2), 627.

30.   Nachit, M. M.; Elouafi, I.; Pagnotta, M. A.; El Saleh, A.; Iacono, E.; Labhilili, M.; Asbati, A.; Azrak, M.; Hazzam, H.; Benscher, D.; Khairallah, M.; Ribaut, J. M.; Tanzarella, O. A.; Porceddu, E.; Sorrells, M. E., Molecular linkage map for an intraspecific recombinant inbred population of durum wheat *Theor. Appl. Genet.* **2001,** 102, 177-186.

31.   Bayles, B. B.; Clark, J. A., Classification of wheat varieties grown in the United States in 1949 In USDA, Ed. 1954; p 173.

32.   Adom, K. K.; Sorrells, M. E.; Liu, R. H., Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. *J Agric Food Chem* **2005,** 53, (6), 2297-306.

33.   Dewanto, V.; Wu, X.; Liu, R. H., Processed sweet corn has higher antioxidant activity. *J Agric Food Chem* **2002,** 50, (17), 4959-64.

34.   Liu, C. S.; Glahn, R. P.; Liu, R. H., Assessment of Carotenoid Bioavailability of Whole Foods Using a Caco-2 Cell Culture Model Coupled with an in Vitro Digestion. *J. Agric. Food Chem.* **2004,** 52, (13), 4330-4337.

35.   Wolfe, K. L.; Kang, X.; He, X.; Dong, M.; Zhang, Q.; Liu, R. H., Cellular Antioxidant Activity of Common Fruits. *J. Agric. Food Chem.* **2008,** 56, (18), 8418-8426.

36.   Wolfe, K. L.; Liu, R. H., Cellular Antioxidant Activity (CAA) Assay for Assessing Antioxidants, Foods, and Dietary Supplements. *J Agric Food Chem* **2007**.

37.   Liu, R. H., Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *Am J Clin Nutr* **2003,** 78, (3 Suppl), 517S-520S.

38.   Liyana-Pathirana, C. M.; Shahidi, F., Importance of Insoluble-Bound Phenolics to Antioxidant Properties of Wheat. *J. Agric. Food Chem.* **2006,** 54, (4), 1256-1264.

39.   Mattila, P.; Pihlava, J. M.; Hellstrom, J., Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. *J Agric Food Chem* **2005,** 53, (21), 8290-5.

40.   Sosulski, F.; Krygier, K.; Hogge, L., Free, Esterified, and Insoluble-Bound Phenolic-Acids .3. Composition of Phenolic-Acids in Cereal and Potato Flours. *Journal of Agricultural and Food Chemistry* **1982,** 30, (2), 337-340.

41.   Siebenhandl, S.; Grausgruber, H.; Pellegrini, N.; Del Rio, D.; Fogliano, V.; Pernice, R.; Berghofer, E., Phytochemical profile of main antioxidants in different fractions of purple and blue wheat, and black barley. *Journal of Agricultural and Food Chemistry* **2007,** 55, (21), 8541-8547.

42.   Li, L.; Shewry, P. R.; Ward, J. L., Phenolic acids in wheat varieties in the HEALTHGRAIN Diversity Screen. *J Agric Food Chem* **2008,** 56, (21), 9732-9.

43.   Panfili, G.; Fratianni, A.; Irano, M., Normal phase high-performance liquid chromatography method for the determination of tocopherols and tocotrienols in cereals. *J Agric Food Chem* **2003,** 51, (14), 3940-4.

44.   Hidalgo, A.; Brandolini, A.; Pompei, C.; Piscozzi, R., Carotenoids and tocols of einkorn wheat (Triticum monococcum ssp monococcum L.). *Journal of Cereal Science* **2006,** 44, (2), 182-193.

45.   Lampi, A. M.; Nurmi, T.; Ollilainen, V.; Piironen, V., Tocopherols and Tocotrienols in Wheat Genotypes in the HEALTHGRAIN Diversity Screen. *Journal of Agricultural and Food Chemistry* **2008,** 56, (21), 9716-9721.

46.   Grela, E. R., Nutrient composition and content of antinutritional factors in spelt (Triticum spelta L) cultivars. *Journal of the Science of Food and Agriculture* **1996,** 71, 399-404.

47.   Fardet, A.; Rock, E.; Remesy, C., Is the in vitro antioxidant potential of whole-grain cereals and cereal products well reflected in vivo? *Journal of Cereal Science: 48 (2) 258-276* **2008,** 48, (2), 258-276.

CHAPTER 3

THE GERM FRACTION OF WHOLE WHEAT IS A GOOD CANDIDATE FOR A

FUNCTIONAL INGREDIENT IN WHEAT-BASED FOOD PRODUCTS

Neal Okarter, Chang-Shu Liu, Lynn Haynes, and Rui Hai Liu

Abstract

The objective of this study was to determine the phytochemical content, antioxidant activity, and antiproliferative activity of a commercial blend of whole wheat and its milled fractions, the bran/shorts, germ, and endosperm. The total phenolic content of the germ, bran/shorts, whole seed, and endosperm were 2140, 2020, 897, and 307 µmol gallic acid equivalents/100 g DW, respectively. Ferulic acid was the predominant phenolic acid found in whole wheat and its milled fractions. *p*-Coumaric acid, syringic acid, *p*-hydroxybenzoic acid, and caffeic acid were also detected. The total carotenoid content of the germ, whole seed, endosperm, and bran/shorts was 1016, 274, 263, and 233 µg/100 g DW, respectively. Lutein was the predominant carotenoid found in whole wheat and its milled fractions. The bran/shorts fraction consisted mainly of tocotrienols, though the germ fraction consisted mainly of tocopherols. The whole seed contained significantly more tocotrienols than the endosperm, though the tocopherol content of the whole seed and endosperm was similar. The Oxygen Radical Absorbance Capacity (ORAC) assay was used to determine the antioxidant activity of whole wheat and its milled fractions. The germ had the highest total ORAC with 18380 µmol Trolox equivalents/100 g DW, followed by the bran/shorts, whole seed, and endosperm with 16487, 7133, and 2583 µmol TE/100 g DW, respectively. Free phenolics extracts from the germ significantly ($p < 0.01$) inhibited the proliferation of HepG2 cells after 96 hours at

concentrations of 10 mg/mL, though free phenolics extracts from the whole seed, bran/shorts fraction, and endosperm had no antiproliferative activity. Insoluble-bound phenolics extracts from whole wheat and its milled fractions significantly ($p < 0.01$) inhibited the proliferation of HepG2 cells after 96 hours at 10 mg/mL. The germ fraction had high total phenolic content, high phenolic acid content, high tocopherol content, high antioxidant activity, and ability to inhibit the proliferation of HepG2 cell cultures after 96 hours. Therefore, the germ fraction may be a good candidate for a functional ingredient in wheat-based food products.

3.1 Introduction

A whole grain consists of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components - the starchy endosperm, germ and bran - are present in the same relative proportions as they exist in the intact caryopsis (*1*). The endosperm, the largest component of the whole seed, contains the food supply for the germ and provides energy for the rest of the plant. The bran is the multi-layered outer skin of the grain that protects the germ and the endosperm from damage due to sunlight, pests, water, and diseases. The shorts are a combination of the bran layer and the endosperm that are formed as a result of milling the whole seed. The germ is the embryo, the part of the grain that becomes a new plant when fertilized by pollen. However, the industry definition of germ refers to the embryonic axis of the germ not including the scutellum, which contains more vitamins and fat than the embryo (*2*).

The health benefits of whole grain and dietary fiber consumption with regards to reduced risk of chronic diseases including cardiovascular disease, type II diabetes, and cancer have been reviewed in the literature (*3, 4*). The health benefit of whole

grain consumption is attributed to phytochemicals, naturally occurring, non-nutrient, bio-active, plant compounds found in fruits, vegetables, and whole grains (*5*).

Wheat is the prominent grain based on consumption. Hard red winter wheat cultivars are most popular due to high gluten content, making them particularly good for cookies and some bread (*6*). White wheat cultivars, both hard and soft, are often exported from North America for use in making flat breads and noodles (*7*). Commercial blends of whole wheat are used in the production of wheat-based food products.

Grain processing is a necessary step in the production of grain products. The processing of grains includes but is not limited to milling and extrusion. The processing of grains may also influence the amounts of phytochemicals found in grains (*8, 9*). Milling is the first processing step in the manufacturing of food products. Milling involves the separation of the germ and bran from the endosperm so that the endosperm can be ground into refined flour. Prior to milling, the grain is often tempered in order to ease the removal of the germ and bran from the endosperm. Tempering significantly lowered the phytic acid content of wheat in a time-dependent manner (*10*). The milling of the whole seeds generally results in the reduction of macro- and micro-nutrients (*11*). The decreases in total phenolic content, phenolic composition and antioxidant activity due to processing create the need for functional ingredients in refined wheat products so that processed grain products maintain the same nutritional quality as the whole grain.

Phytochemical content of whole wheat and its milled fractions has been previously reported (*8, 12, 13*). However, the phytochemical content of a commercial blend of whole wheat and its milled fractions has not been reported. The objective of this study was to determine the phytochemical content, antioxidant activity, and

antiproliferative activity of a commercial blend of whole wheat and its milled fractions, the bran/shorts, germ, and endosperm.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Sodium hydroxide, sodium sulfate, and hexanes were purchased from Fisher Scientific (Pittsburgh, PA). Acetone, sodium carbonate, sodium chloride, ethanol, and ethyl acetate were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Folin-Ciocalteu reagent, ferulic acid, p-coumaric acid, caffeic acid, p-hydroxybenzoic acid, carotene (α:β, 1:2) , vanillic acid, syringic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), pyrogallol, quercetin dihydrate, kaempferol, catechin hydrate, and lutein were purchased from Sigma (St. Louis, MO). Gallic acid was purchased from ICN Biomedicals, Inc. (Aurora, OH). 2,2'-Azobis (2-amidinopropane) dihydrochloride (ABAP) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Zeaxanthin and β-cryptoxanthin were purchased from Indofine Chemical Company Inc. (Hillsborough, NJ). All tocopherols and tocotrienols were purchased from Calbiochem (Darmstadt, Germany). Methylene blue was purchased from BBL (Cockeysville, MD). HepG2 cell cultures were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). Williams' Medium E (WME) and Hanks' Balanced Salt Solution (HBSS) were purchased from Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). All reagents used in the study were analytical or reagent grade.

3.2.2 Preparation of Samples

A commercial blend of soft red wheat was obtained from Lynn Haynes at Kraft Foods (East Hanover, NJ) and milled into three fractions; the germ fraction, the endosperm fraction, and the bran/shorts fraction. Milling yields were used to determine the percent contribution to the whole seed and were confirmed by ashing the samples according to the AOAC method (*14*). The whole seed was separately milled to whole grain flour. Samples were protected from light and stored at -20 ˚C until extraction and analysis.

3.2.3 Extraction of Free Phenolics

Free phenolics of wheat samples were extracted using a modification of the method previously reported by Adom *et al* (*15, 16*). Briefly, one gram of whole-wheat flour, endosperm, bran, or germ was blended with 50 mL of 80% chilled acetone. The mixture was then centrifuged at 2500 G for 10 minutes. The supernatant was removed and the remaining pellet was again extracted with 50 mL of 80% chilled acetone. The mixture was again centrifuged at 2500 G for 10 minutes. The supernatants were pooled and evaporated at 45ºC to dryness. The remaining residue was then reconstituted in methanol/hydrochloric acid (1 M; 85:15, v/v) or deionized water, filtered through a 0.45 μm filter, and stored at -40 ºC until analysis.

3.2.4 Extraction of Insoluble-Bound Phenolics

Insoluble-bound phenolics of wheat samples were extracted using a modification of the method previously reported by our laboratory (*15, 16*). Briefly, insoluble -bound phenolics were extracted from the residue from the free phenolic extraction. The residue was first digested with 2 M sodium hydroxide at room temperature for 1 h while shaking under nitrogen. The mixture was then neutralized

with concentrated hydrochloric acid. Hexanes were used to extract lipids in the mixture. The remaining mixture was then extracted five times with ethyl acetate. The ethyl acetate fractions were pooled and evaporated to dryness. The remaining residue as reconstituted in methanol/hydrochloric acid (1 M; 85:15, v/v) or deionized water, filtered through a 0.45 μm filter, and stored at -40 ºC until analysis.

3.2.5 Extraction of Soluble-Conjugated Phenolic Compounds

Soluble-conjugated phenolic compounds were extracted using the method routinely used in our laboratory (*15*). Briefly, free phenolics extracts were digested with 2 M sodium hydroxide for 1 h at room temperature under nitrogen gas, and then neutralized with concentrated hydrochloric acid. The mixtures were then extracted five times with ethyl acetate. The ethyl acetate fractions were pooled and evaporated to dryness under nitrogen at 35˚C. The remaining residues were dissolved in methanol/hydrochloric acid (1 M; 85:15, v/v), filtered through a 0.45 μm filter, and stored at -40 ºC until analysis.

3.2.6 Determination of Total Phenolic Content

Total phenolic content was determined using the colorimetric method described by Singleton *et al* (*17*) and modified in our lab (*18*). Briefly, extracts were reacted with Folin-Ciocalteu reagent and then neutralized with sodium carbonate. After 90 minutes, the absorbance of the resulting solution was measured at 760 nm. Gallic acid was used as the standard and total phenolic content was expressed as μmol GAE/100 g DW.

3.2.7 Determination of Phenolic Composition

The determination of the phenolic composition was conducted using an rp-HPLC-DAD method. Briefly, the mobile phase [water at pH 2.8 with acetic acid (A) and acetonitrile/water 70:30 v/v to pH 2.8 with acetic acid (B)] was delivered using a Waters 600E quaternary pump at a flow of 1.5 mL/min using the following gradient: 0/100% – 10/90% B/A for 2.5 minutes, 10/90% – 12/88% B/A for 2.5 minutes, 12/88% – 23/77% B/A for 10 minutes, 23/77% – 95/5% B/A for 4 minutes, and 95/5% – 0/100% B/A for 4 minutes. Total run time for each injection was 25 minutes. Seventy five microliters sample were injected using a Water 717 Autosampler. Seperation of phenolic compounds was done using a C18 5 μm, 250 mm x 4.6 mm column (Grace Vydac, Baltimore, MD). Phenolic compounds were detected using a Waters 996 Photodiode Array Detector. Each injection was monitored at 315 nm. *p*-Hydroxybenzoic was quantified at 254 nm. Catechin and syringic acid were quantified at 275 nm. Ferulic acid, *p*-coumaric acid, salicylic acid, and caffeic acid were quantified at 315 nm. Quercetin, and kaempferol were quantified at 365 nm. Detection limits were determined using a previously reported method (*19*) and were all less than 0.19 and 0.75 μg per injection for phenolic acids and flavonoids, respectively. Percent recoveries for all hydroxybenzoic acid derivatives, hydroxycinnamic acid derivatives, and flavonoids were greater than 72%, 81%, and 90%, respectively. Data signals were acquired and processed using Waters Empower software (2002) (Waters Corp., Milford, MA).

3.2.8 Extraction of Carotenoids

Carotenoids were extracted using a modified method previously described by our laboratory (*8, 20*). The extraction was performed under dim lighting and all sample tubes were wrapped in lightproof paper to protect carotenoids from light-

induced degradation. Samples and 60 mg magnesium carbonate were placed in a 10 x 120 mm screw cap test tube to which 3 mL methanol/tetrahydrofuran (1:1, v/v) were added. The sample was vortexed, placed in a 75 ºC water bath for 5 min, vortexed again and immediately centrifuged at 2000 G for 5 minutes. After the upper solvent layer was removed to a second test tube, three additional washes of 3 mL methanol/tetrahydrofuran (1:1, v/v) were performed for complete extraction of carotenoids. Upper solvent extraction layers were pooled and vortexed with 1.5 g sodium sulfate. The solvent layer was removed and the residue was rinsed at least two times with 2 mL hexane. The combined hexane solvent was evaporated to dryness with nitrogen. The dry residue was re-dissolved with 0.5 mL methanol/tetrahydrofuran (1:1, v/v) stored under nitrogen at -20 ºC until HPLC analysis.

3.2.9 Determination of Carotenoid Content

Carotenoid content was determined using the method routinely used in our laboratory (*20, 21*). Briefly, the carotenoid content of each sample was determined using an rp-HPLC procedure employing a 250×4.6 mm YMC C30 column, 3 μm particle size (YMC, Waters Inc., Wilmington, NC). Methanol/water (95:5, v/v, A) and methyl tert-butyl ether (B) were delivered at 1.9 mL/minute using a Water 515 HPLC pump (Water Corp., Milford, MA). The gradient procedure was as follows: 25% B for 11 minutes, 25 – 30 % B for 2 minutes, 30% B for 9 minutes, 30 – 25% B for 1 minute, and 25% B for at least 5 minutes where the next injection would occur. A Waters 2487 dual wavelength absorbance detector (Waters Corps, Miford, MA) was used for UV detection of analytes at 450 nm. Data signals were acquired and processed on a PC running the Waters Millennium software, version 3.2 (1999) (Waters Corp, Miford, MA). Percent recoveries for all carotenoids were greater that 94%. The carotenoid content of each sample extract was extrapolated from a pure

carotenoid standard curve. All samples were injected via a 20 µL loop using a 50 µL syringe and peak heights were used for all calculations. Data were expressed as µg/100 g DW.

3.2.10 Extraction of Vitamin E

Extraction of tocopherols and tocotrienols from whole wheat was conducted using a modified method described previously (*21, 22*). Briefly, 2 mL 95% ethanol, 1 mL 300 mM sodium chloride, 4 mL 500 mM pyrogallol in ethanol, 1 mL 1 M ascorbic acid, then 2 mL 10.7 M potassium hydroxide were added to 1 g whole wheat flour, endosperm, or bran, or 300 mg germ in a glass screw-cap tube. The headspace was flushed with nitrogen and the sample was digested for 45 minutes at 75°C in a constantly agitated water bath. After 45 minutes, the tubes were cooled to room temperature in an ice bath. After addition of 750 µL 3 M sodium chloride and 8 mL hexanes/ethyl acetate (9:1, v/v), the solution was then sonicated for 10 minutes, and then vitamin E was extracted for 5 minutes. The tube was then centrifuged at 100 G for 1 minute and the organic layer was removed. The extraction procedure was repeated twice and the organic layers from each extraction were pooled. The pooled organic layers were transferred to a separatory funnel and were washed twice with 10 mL distilled water. After transfer to an Erlenmeyer flask, the solution was dehydrated with 5 g sodium sulfate. The solution was then filtered using glass wool and evaporated to dryness under a gentle stream of nitrogen at 35 ˚C. The residue was dissolved in 2 mL hexanes/ethyl acetate/acetic acid (99.0:0.5:0.5, v/v/v), filtered through a 0.45 µm filter, capped under nitrogen, and stored at -20°C until HPLC analysis.

3.2.11 Determination of Vitamin E Content

Chromatographic separation of tocopherols and tocotrienols was done using np-HPLC with a fluorescence detector. Briefly, the mobile phase, hexanes/ethyl acetate/acetic acid (99.0:0.5:0.5, v/v/v) was filtered using a 0.45 μm filter and delivered by a Waters 501 Solvent Delivery System at 1 mL/min. One hundred microliters of sample were injected into the system and a Grace Vydac 250 x 4.6 mm, 5 μm particle size silica column (Grace Vydac Carlsbad, CA) was used to separate each form of vitamin E. Detection of tocopherols and tocotrienols was conducted using fluorescence detection with a Waters 474 Scanning Fluorescent Detector at an excitation wavelength of 290 nm and an emission wavelength of 330 nm. Data signals were acquired and processed on a PC running the Waters Millennium Software, version 3.2 (1999) (Waters Corp, Miford, MA). Samples were injected using a 200 μL syringe. The detection limits for α-tocopherol and α-tocotrienol were 0.1 ng per injection. The percent recovery for α-tocopherol and α-tocotrienol were 95.2 ± 5.3 and 99.9 ± 9.9, respectively. The detection limits and percent recoveries for all other forms of vitamin E were assumed to be on the same order.   Total tocopherol and tocotrienol content was expressed as μg/g DW.

3.2.12 Antioxidant Activity

The antioxidant activity was determined using oxygen radical absorbance capacity (ORAC) assay described by Huang *et al* (*23*) and modified in our lab (*24, 25*) was used . Phenolic extract dilutions were prepared with 75 mM phosphate buffer (pH 7.4). The assay was performed in black-walled 96-well plates (Corning Scientific, Corning, NY). The outside wells of the plate were not used as there was much more variation from them than from the inner wells. Each well contained 20 μL extract or 20 μL Trolox standard (range 6.25 – 50 μM), and 200 μL fluoroscein (final

concentration 0.96 µM), which were incubated at 37 ˚C for 20 minutes. After incubation, 20 µL 119 mM ABAP was added to each well. Fluorescence intensity was measured using Fluoroskan Ascent FL plate-reader (Thermo Labsystems, Franklin, MA) at excitation of 485 nm and emission of 520 nm for 35 cycles. Oxygen radical absorbance capacity was expressed as µmol TE/100 g DW.

3.2.13 Cell Culture

HepG2 cells were grown in growth medium (WME supplemented with 5% FBS, 10 mM HEPES Buffer to pH 7.4, 2 mM L-glutamine, 5 µg/mL insulin, 0.05 µg/mL hydrocortisone, 50 units/mL penicillin, 50 µg/mL streptomycin, and 100 µg/mL gentamicin) and were maintained at 37ºC and 5% CO_2 as described previously (*26, 27*).

3.2.14 Antiproliferative Activity

The antiproliferative activity of phenolics extracts from whole wheat and its milled fractions toward HepG2 human liver cancer cell growth were measured using the previously described methylene blue assay (*28*). HepG2 cells were plated at a density of 2.5 x 10^4 cells per well and kept at 37˚C in 5% CO_2 for 4 hours. The medium was then replaced by medium containing the wheat fraction extracts and the cells were incubated at 37 ˚C in 5% CO_2 for 96 hours. The medium was removed and cells were rinsed once with PBS and treated with a fixing/staining solution containing 0.6% methylene blue (BBL, Cockeysville, MD) and 1.25% glutaldehyde (Sigma-Aldrich, St. Louis, MO) in Hank's Balanced Salt Solution (Invitrogen) at 37˚C for 1 hour. After the fixing/staining solution was removed, the cells were rinsed with water and dried. Methylene blue stain was eluted with an elution solution containing ethanol/PBS/acetic acid 50:49:1 v/v/v by agitating the plate for 30 minutes. The

absorbance was measured by a Dynex Technologies MRX-II microplate reader (Dynex Technologies Inc, Chantilly, VA) at 570 nm.

3.2.15 Cytotoxicity

The cytotoxicity of extracts toward HepG2 cells was measured using the method described previously (*28, 29*). HepG2 cells were plated at a density of 4 x 10^4 cells per well and kept at 37˚C in 5% CO_2 for 4 hours. The medium was then replaced by medium containing the wheat fraction extracts and the cells were incubated at 37 ˚C in 5% CO_2 for 24 hours. The medium was removed and cells were rinsed once with PBS and treated with a fixing/staining solution containing 0.6% methylene blue (BBL, Cockeysville, MD) and 1.25% glutaldehyde (Sigma-Aldrich, St. Louis, MO) in Hank's Balanced Salt Solution (Invitrogen) at 37˚C for 1 hour. After the fixing/staining solution was removed, the cells were rinsed with water and dried. Methylene blue stain was eluted with an elution solution containing ethanol/PBS/acetic acid 50:49:1 v/v/v by agitating the plate for 30 minutes. The absorbance was measured by a Dynex Technologies MRX-II microplate reader (Dynex Technologies Inc, Chantilly, VA) at 570 nm.

3.2.15 Statistical Analysis

Data were reported as mean ± standard deviation (SD) for triplicate determinations of each sample. ANOVA, Tukey's comparison test, and regression analysis were performed using Minitab Statistical Software version 15 (State College, PA). Statistical significance was defined to be at a level of $p < 0.05$.

3.3 Results

3.3.1 Total Phenolic Content

The total phenolic content of the free and insoluble-bound fractions of whole wheat and its milled fractions were determined using the Folin-Ciocalteau Reagent. The free phenolic content of the germ, bran/shorts, whole seed, and endosperm were 1620, 653, 348, and 187 μmol GAE/100 g DW, respectively (**Figure 3.1**). The free phenolic contents of the endosperm and the whole seed were statistically similar ($p > 0.05$). The insoluble-bound phenolic content of the bran/shorts, whole seed, germ, and endosperm, and whole seed were 1360, 549, 510, and 120 μmol GAE/100 g DW, respectively (**Figure 3.1**). The total phenolic contents of the germ and the whole seed were statistically similar ($p > 0.05$). The total phenolic content, sum of the free and insoluble-bound fractions, of the bran/shorts, germ, endosperm, and whole seed were 2140, 2020, 897, and 307 μmol GAE/100 g DW, respectively (**Figure 3.1**). The total phenolic contents of the bran/shorts and the germ were statistically similar ($p > 0.05$) though the total phenolic contents of the endosperm and the whole seed were statistically different ($p < 0.05$).

The bran/shorts contributed the most to the free phenolic content of the reconstituted whole seed (49.1%), followed by the endosperm (41.1%), and the germ (9.8%). The bran/shorts contributed the most to the insoluble-bound phenolic content of the reconstituted whole seed (77.7%), followed by the endosperm (20.0%), and the germ (2.3%). The bran/shorts contributed the most to the total phenolic content of the reconstituted whole seed (65.4%), followed by the endosperm (29.1%), and the germ (5.5%).
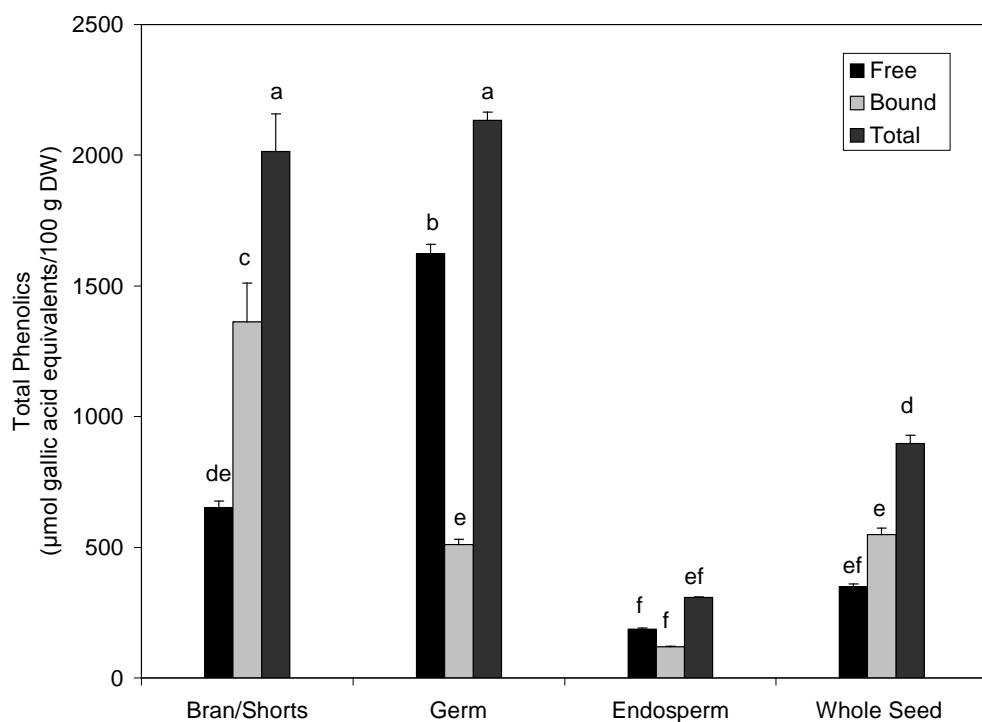
Figure 3.1 – Total phenolic content of whole wheat and its milled fractions. Values expressed as μmol gallic acid equivalents/100 g DW (mean ± standard deviation, n = 3). Bars with no letters in common are significantly different *(p < 0.05)*.

3.3.2 Phenolic Acid Composition

The phenolic acid content of the free, soluble-conjugated, and insoluble-bound fractions of whole wheat and its milled fractions was determined using an rp-HPLC-DAD method. Ferulic acid was the predominant phenolic acid found in whole wheat and its milled fractions. Other phenolic acids were also found in whole wheat and its milled fractions. *p*-Coumaric acid was the second most predominant phenolic acid found in whole wheat and its milled fractions. Syringic acid and caffeic acid were found in the whole seed, bran/shorts, and germ, but not in the endosperm. Syringic acid was found in the insoluble-bound fraction of the bran/shorts and whole seed, but not in the germ. All of the caffeic acid found in the bran/shorts and whole seed was found in the insoluble-bound fraction. All of the caffeic acid found in the germ was found in the free fraction. *p*-Hydroxybenzoic acid was only found in the free fraction of the germ (108.7 ± 10.2 μmol/100 g DW). No flavonoids (catechin, quercetin, or kaempferol) were detected in the whole seed or any of its milled fractions.

The free ferulic acid content of the germ was 367.0 μmol/100 g DW. The free ferulic acid content of the bran/shorts, whole seed and endosperm was 29.3, 16.2, and 10.8 μmol/100 g DW, respectively. The free ferulic acid content of the germ fraction was significantly greater ($p < 0.05$) than the free ferulic acid content of the other milled fractions. The soluble-conjugated ferulic acid content of the germ, bran/shorts, whole seed, and endosperm was 157.8, 72.9, 12.7, and 9.2 μmol/100 g DW, respectively. The ferulic acid content of the germ was significantly higher than the soluble-conjugated ferulic acid content of each milled fraction ($p < 0.05$). The soluble-conjugated ferulic acid content of the endosperm and whole seed were not significantly different ($p > 0.05$). The insoluble-bound ferulic acid content of the bran/shorts was 600.7 μmol/100 g DW. The insoluble-bound ferulic acid content of the germ and whole seed were 253.3 and 264.7 μmol/100 g DW, respectively. The

endosperm had the lowest amount of insoluble-bound ferulic acid with 34.5 μmol/100 g DW. The germ and bran/shorts contained the most total ferulic acid (778 and 703 μmol/100 g DW) followed by the whole seed, and endosperm with 289.5, and 54.4 μmol/100 g DW, respectively (**Table 3.1**). The total ferulic acid content of the germ is not significantly different from the total ferulic acid content of the bran/shorts (p > 0.05).

The free ferulic acid content from the germ fraction contributed 47.2% to the total ferulic acid content of the germ. The free ferulic acid content from the endosperm contributed 20.0% to the total ferulic acid content of the endosperm, whereas the free ferulic acid content of the bran/shorts and whole seed both contribute 4.2% to the total ferulic acid content of each milled fraction. The soluble-conjugated ferulic acid content from the germ fraction contributed 20.3% to the total ferulic acid content of the germ. The insoluble-bound fraction from the whole seed, the bran/shorts, and the endosperm contributed 91.4, 85.4, and 63.4% to the total ferulic acid content, respectively.

The insoluble-bound fraction from the germ contributed 6.3% to the total *p*-coumaric acid content. The free *p*-coumaric acid content of the germ, bran/shorts, whole seed, and endosperm was 165.9, 15.3, 9.7, and 7.7 μmol/100 g DW. The free *p*-coumaric acid content of the germ was significantly higher (p < 0.05) than that of the bran/shorts, endosperm, and whole seed. The soluble-conjugated *p*-coumaric acid content of the bran/shorts, germ, and whole seed was 10.9, 9.5, and 7.5 μmol/100 g DW. No soluble-conjugated *p*-coumaric acid was found in the endosperm. The soluble-conjugated *p*-coumaric acid contents of the bran/shorts and the whole seed were significantly different (p < 0.05). The insoluble-bound *p*-coumaric acid content of the bran/shorts, whole seed, germ and endosperm were 29.8, 14.9, 11.8, and 7.1 μmol/100 g DW, respectively. All of the insoluble-bound *p*-coumaric acid contents

were statistically different ($p < 0.05$). The total *p*-coumaric acid content of the germ, bran/shorts, whole seed, and endosperm were 187.2, 56.0, 32.1, and 14.8 µmol/100 g DW (Table 3.1). The total *p*-coumaric acid content of the endosperm and whole seed were statistically similar ($p > 0.05$).

The free fraction from the germ contributed the most to the total *p*-coumaric acid content of the germ, 88.6%, though the free fraction of the endosperm, whole seed, and bran/shorts contributed 52.0, 30.2, and 27.3% to the total *p*-coumaric acid content of each milled fraction, respectively. The soluble-conjugated *p*-coumaric acid content of the whole seed, bran/shorts, and germ contributed 23.4, 19.5, and 5.1% to the total *p*-coumaric acid content of each milled fraction, respectively. The insoluble-bound fraction of the bran/shorts, endosperm, and whole seed contributed the most to the total *p*-coumaric acid of each milled fraction, 53.2, 48.0, and 46.4%, respectively.

The free syringic acid content of the bran/shorts, and the germ was 16.3 and 5.0 µmol/100 g DW, respectively. The free syringic acid contents of the bran/shorts and the germ were statistically different ($p < 0.05$). Syringic acid was found in the insoluble-bound fraction of the bran/shorts and whole seed, but not in the germ. The soluble-conjugated syringic acid content of the bran/shorts, and the whole seed was 20.1 and 5.9 µmol/100 g DW, respectively. Both contents were significantly different ($p < 0.05$) from each other. The insoluble-bound syringic acid content of the bran/shorts, and the whole seed was 7.5 and 5.1 µmol/100 g DW, respectively. Both contents were significantly different ($p < 0.05$) from each other. The total syringic acid content of the bran/shorts, germ, and whole seed was 43.9, 11.0, and 5.0 µmol/100 g DW, respectively (**Table 3.1**). All of the total syringic acid contents were significantly different ($p < 0.05$).

The free syringic acid content from the bran/shorts contributed 37.1% to the total syringic acid content of the fraction while the free syringic acid content from the

Table 3.1 – Phenolic acid composition of a commercial blend of whole wheat and its milled fractions. Values expressed as µmol/100 g DW (mean ± SD, n = 3). Percent contribution to the total in each milled fraction and whole wheat is in parentheses. Values with no letters in common within each column are significantly different (p < 0.05). nd – not detected

	Free	Soluble-Conjugated	Insoluble-Bound	Total
		Ferulic Acid		
Bran/Shorts	29.3 ± 5.0 b (4.2%)	72.9 ± 12.8 b (10.4%)	600.7 ± 55.3 a (85.4%)	702.9 ± 61.1 a
Germ	367.0 ± 84.2 a (47.2%)	157.8 ± 3.6 a (20.3%)	253.3 ± 13.4 b (32.6%)	778.1 ± 74.2 a
Endosperm	10.8 ± 0.4 b (19.9%)	9.2 ± 0.8 c (16.9%)	34.5 ± 3.6 c (63.4%)	54.4 ± 4.6 c
Whole Seed	12.2 ± 1.7 b (4.2%)	12.7 ± 2.6 c (4.4%)	264.7 ± 14.1 b (91.4%)	289.5 ± 14.3 b
		p-Coumaric Acid		
Bran/Shorts	15.3 ± 2.1 b (27.3%)	10.9 ± 2.0 a (19.5%)	29.8 ± 1.6 a (53.2%)	56.0 ± 4.7 b
Germ	165.9 ± 27.2 a (88.6%)	9.5 ± 0.3 ab (5.1%)	11.8 ± 0.5 c (6.3%)	187.2 ± 27.1 a
Endosperm	7.7 ± 0.1 b (52.0%)	nd	7.1 ± 0.1 d (48.0%)	14.8 ± 0.2 c
Whole Seed	9.7 ± 0.4 b (30.2%)	7.5 ± 0.2 b (23.4%)	14.9 ± 0.3 b (46.4%)	32.1 ± 0.5 c

Table 3.1 (*continued*)

	Free	Soluble-Conjugated	Insoluble-Bound	Total
		Syringic Acid		
Bran/Shorts	16.3 ± 3.1 a (37.1%)	20.1 ± 2.8 a (45.8%)	7.5 ± 1.8 a (17.1%)	43.9 ± 4.0 a
Germ	5.0 ± 0.3 b (100%)	nd	nd	5.0 ± 0.3 c
Endosperm	nd	nd	nd	nd
Whole Seed	nd	5.9 ± 0.2 b (53.6%)	5.1 ± 0.1 b (46.4%)	11.0 ± 0.2 b
		Caffeic Acid		
Bran/Shorts	nd	nd	8.6 ± 0.7 a (100%)	8.6 ± 0.7 a
Germ	3.2 ± 0.2 (100%)	nd	nd	3.2 ± 0.2 b
Endosperm	nd	nd	nd	nd
Whole Seed	nd	nd	3.3 ± 0.1 b (100%)	3.3 ± 0.1 b

germ contributed entirely to the total syringic acid content of the germ. The soluble-conjugated syringic acid content from the whole seed contributed 53.6% to the total syringic acid content of the fraction while the soluble-conjugated syringic acid content from the bran/shorts contributed 45.8% to the total syringic acid content of the bran/shorts. The insoluble-bound syringic acid content from the whole seed contributed 46.4% to the total syringic acid content of the fraction while the insoluble-bound syringic acid content from the bran/shorts contributed 17.1% to the total syringic acid content of the bran/shorts.

The total caffeic acid content of the bran/shorts, whole seed, and germ was 8.6, 3.3, and 3.2 μmol/100 g DW, respectively (**Table 3.1**). The total caffeic acid contents of the whole seed and the germ were statistically similar ($p > 0.05$).

3.3.3 Carotenoid Content

The carotenoid content of whole wheat and its milled fractions was determined using an rp-HPLC method. Lutein was the most abundant carotenoid in the whole seed. Other carotenoids, zeaxanthin, β-cryptoxanthin and β-carotene, were also detected in whole wheat and its milled fractions. No α-carotene was found in whole wheat or its milled fractions. The total carotenoid content was determined to be the sum of the lutein, zeaxanthin, β-cryptoxanthin and β-carotene contents.

The lutein content of the germ, whole seed, endosperm, and bran/shorts was 394, 163, 161, and 120 μg/100 g DW, respectively (**Table 3.2**). The lutein content of the germ was significantly higher than that of the whole seed, endosperm and bran/shorts. The endosperm contributed 76% to the total lutein content of the reconstituted whole seed, followed by the bran/shorts and the germ with 19 and 5%, respectively.

Both zeaxanthin and β-carotene were found in similar amounts in the germ fraction, 287 and 284 μg/100 g DW, respectively (**Table 3.2**). However, the content of β-carotene found in the whole seed, endosperm, and bran/shorts is significantly higher ($p < 0.05$) than the zeaxanthin content found in the whole seed, endosperm, and bran/shorts. The β-carotene content of the whole seed, endosperm, and bran/shorts was 86, 84, and 63 μg/100 g DW, respectively. The zeaxanthin content of the bran/shorts, whole seed, and endosperm was 38, 17, and 5 μg/100 g DW, respectively. The endosperm contributed 74% to the total β-carotene content of the reconstituted whole seed, followed by the bran/shorts and the germ with 19 and 7%, respectively. The bran/shorts contributed 51% to the total zeaxanthin content of the reconstituted whole seed, followed by the germ and the endosperm with 30 and 19%, respectively.

The β-cryptoxanthin content of the germ, endosperm, bran/shorts, and whole seed was 51, 13, 10, and 9 μg/100 g DW, respectively (**Table 3.2**). The endosperm contributed 24% to the total β-cryptoxanthin content of the reconstituted whole seed, followed by the bran/shorts and the germ with 20 and 8%, respectively.

The total carotenoid content of the germ, whole seed, endosperm, and bran/shorts was 1016, 274, 263, and 233 μg/100 g DW, respectively (**Table 3.2**). The total carotenoid content of the germ was significantly higher than that of the whole seed, endosperm and bran/shorts. The endosperm contributed 71% to the total carotenoid content of the reconstituted whole seed, followed by the bran/shorts and the germ with 21 and 8%, respectively.

3.3.4 Vitamin E Content

The tocopherol and tocotrienol content of whole wheat and its milled fractions were determined using an np-HPLC-FD method. Only α and β forms of vitamin E were detected in the whole seed and its milled fractions, though trace amounts of γ-

Table 3.2 – Carotenoid content of a commercial blend of whole wheat and its milled fractions. Values expressed as µg/100 g (mean ± SD, n = 3). Percent contribution to the total is in parentheses. Values with no letters in common are significantly different (p < 0.05).

	Lutein	Zeaxanthin	β-Cryptoxanthin	β-Carotene	Total Carotenoid
Bran/Shorts	120 ± 10 d (19%)	38 ± 7 e (51%)	10 ± 3 (20%)	63 ± 25 d (19%)	233 ± 36 cd (21%)
Germ	394 ± 37 b (5%)	287 ± 87 bc (30%)	51 ± 10 d (8%)	284 ± 37 bc (7%)	1016 ± 150 a (8%)
Endosperm	161 ± 12 cd (76%)	5 ± 1 (19%)	13 ± 2 e (72%)	84 ± 28 d (74%)	263 ± 34 bc (71%)
Whole Seed	163 ± 6 cd	17 ± 1 e	9 ± 1 e	86 ± 23 d	274 ± 19 bc

tocopherol were detected in the germ. β-Tocotrienol was the predominant tocotrienol found in all milled fractions and the whole seed.

The total tocopherol content of the germ, bran/shorts, whole seed, and endosperm was 73.3, 6.8, 3.1, and 2.2 µg/g DW, respectively (**Table 3.3**). The total tocopherol content of the germ was significantly higher ($p < 0.05$) than that of all the other milled fractions. The α-tocopherol content of the germ, bran/shorts, whole seed, and endosperm was 46.6, 4.3, 1.8, and 1.3 µg/g DW, respectively. The α-tocopherol content of the germ was significantly higher ($p < 0.05$) than that of all the other milled fractions. The β-tocopherol content of the germ, bran/shorts, whole seed, and endosperm was 25.7, 2.5, 1.4, and 0.9 µg/g DW, respectively. The β-tocopherol content of the germ was significantly higher ($p < 0.05$) than that of all the other milled fractions. The endosperm contributed the most β-tocopherol to the reconstituted whole seed (36.5%) followed by the bran/shorts and the germ with 34.8 and 28.7%, respectively. The bran/shorts contributed the most β-tocotrienol to the reconstituted whole seed (36.6%) followed by the endosperm and the germ with 32.2 and 31.5%, respectively.

The total tocotrienol content of the bran/shorts, whole seed, germ, and endosperm was 47.8, 17.8, 12.9, and 7.7 µg/g DW, respectively (**Table 3.3**). The total tocotrienol content of the bran/shorts was significantly higher ($p < 0.05$) than that of all the other milled fractions. The β-tocotrienol content of the bran/shorts, whole seed, germ, and endosperm was 26.8, 12.6, 7.5, and 6.0 µg/g DW, respectively. The β-tocotrienol content of the bran/shorts was significantly higher ($p < 0.05$) than that of all the other milled fractions. The α-tocotrienol content of the bran/shorts, germ, whole seed, and endosperm was 21.0, 5.4, 5.2, and 1.7 µg/g DW, respectively. The α-tocotrienol content of the bran/shorts was significantly higher ($p < 0.05$) than that of all the other milled fractions. The bran/shorts contributed the most α-tocotrienol to the

Table 3.3 – Vitamin E Content of Whole Wheat and its Milled Fractions. Values expressed as µg/g DW (mean ± SD, n = 3). Percent contribution to the total is in parentheses. Values with no letters in common within each column are significantly different (p < 0.05). nd – not detected

	Tocopherols (µg/g DW)				
	α	β	γ	δ	Total
Bran/Shorts	4.3 ± 0.2 b (36.3%)	2.5 ± 0.1 b (34.8%)	nd	nd	6.8 ± 0.3 b (35.4%)
Germ	46.6 ± 3.0 a (31.5%)	25.7 ± 1.5 a (28.7%)	1.0 ± 0.1 (100%)	nd	73.3 ± 4.6 a (30.5%)
Endosperm	1.3 ± 0.1 b (32.2%)	0.9 ± 0.1 b (36.5%)	nd	nd	2.2 ± 0.03 b (33.5%)
Whole Seed	1.8 ± 0.1 b	1.4 ± 0.3 b	nd	nd	3.1 ± 0.4 b

	Tocotrienols (µg/g DW)				
	α	β	γ	δ	Total
Bran/Shorts	21.0 ± 2.8 a (78.2%)	26.3 ± 2.4 a (60.5%)	nd	nd	47.8 ± 5.2 a (67.1%)
Germ	5.4 ± 0.4 b (1.8%)	7.5 ± 0.3 c (1.4%)	nd	nd	12.9 ± 0.7 bc (1.4%)
Endosperm	1.7 ± 0.3 b (20.0%)	6.0 ± 0.3 c (38.2%)	nd	nd	7.7 ± 0.5 c (31.6%)
Whole Seed	5.2 ± 2.3 b	12.6 ± 0.1 b	nd	nd	17.8 ± 2.4 b

reconstituted whole seed (84.3%) followed by the endosperm and the germ with 20.4 and 1.8%, respectively. The bran/shorts contributed the most β-tocotrienol to the reconstituted whole seed (60.5%) followed by the endosperm and the germ with 38.2 and 1.4%, respectively.

3.3.5 ORAC

The ORAC was expressed as μmol TE/100 g DW. The germ had the highest total ORAC with 18380 μmol TE/100 g DW, followed by the bran/shorts, whole seed, and endosperm with 16487, 7133, and 2583 μmol TE/100 g DW, respectively (**Table 3.4**). The total ORAC of the bran/shorts and the germ were statistically similar ($p >$ 0.05). The germ had the highest free ORAC with 15557 μmol TE/100 g DW, followed by the bran/shorts, whole seed, and endosperm with 5721, 3267, and 2298 μmol TE/100 g DW, respectively. The bran/shorts had the highest insoluble-bound ORAC with 10766 μmol TE/100 g DW, followed by the whole seed, germ, and endosperm with 3866, 2823, and 284 μmol TE/100 g DW, respectively. The insoluble-bound ORAC of the germ and the whole seed were statistically similar ($p >$ 0.05). The total phenolic content was correlated with the ORAC ($R^2 = 0.971$; $p <$ 0.001).

The insoluble-bound fraction of the bran/shorts and whole seed contributed 65.4 and 54.2% to the total ORAC. However, the free fraction of the endosperm and germ contributed more to the total ORAC than the insoluble-bound fraction, 89.0 and 84.2%, respectively.

3.3.6 Cytotoxicity and Cell Proliferation

Free phenolics extracts from the whole seed and its milled fractions did not exhibit any cytotoxic effects in HepG2 cells after 24 hours at up to 10 mg/mL (**Figure

Table 3.4 – ORAC values of whole wheat and its milled fractions. Values expressed as μmol TE/100 g DW (mean ± SD, n = 3). Percent contribution to the total ORAC in each milled fraction and whole wheat is in parentheses. Values with no letters in common are significantly different (p < 0.05).

	Free ORAC	Bound ORAC	Total ORAC[a]
Bran/Shorts	5721 ± 258 d (34.7%)	10766 ± 206 c (65.3%)	16487 ± 178 ab
Germ	15557 ± 1111 b (84.6%)	2823 ± 233 e (15.4%)	18380 ± 1323 a
Endosperm	2298 ± 467 ef (89.0%)	284 ± 14 f (11.0%)	2583 ± 470 e
Whole Seed	3267 ± 468 e (45.8%)	3836 ± 979 e (54.2%)	7133 ± 530 d

[a]Sum of free and insoluble-bound ORAC

3.2). However, phenolics extracts from the insoluble-bound fraction of whole wheat and the bran/shorts fraction exhibited mild cytotoxic effects at 6 mg/mL (Figure 3.3). Phenolics extracts from the insoluble-bound fraction of the endosperm and the germ exhibited cytotoxic effects at 8 mg/mL (**Figure 3.3**).

Free phenolics extracts from the germ inhibited HepG2 cell proliferation to 48.9% of the medium control at a concentration of 10 mg/mL (**Figure 3.2**). Free phenolics extracts from the whole seed, bran/shorts, and endosperm had no inhibitory effects on cell proliferation after 96 hours. Insoluble-bound phenolics extracts from the whole seed and its milled fractions were able to significantly ($p < 0.01$) inhibit the proliferation of HepG2 cells after 96 hours at a dose of 10 mg/mL (**Figure 3.3**). Insoluble-bound phenolics extracts from the bran/shorts, germ, endosperm, and whole seed inhibited HepG2 cell proliferation by 47.1, 53.7, 48.2, and 47.7% of the medium control at a concentration of 10 mg/mL, respectively.

3.4 Discussion

3.4.1 Health Benefits of Whole Grain Phytochemicals

Consumption of whole grains has been associated with reduced risk of cardiovascular disease (*30-32*), type II diabetes (*33-35*), and cancer (*36-38*). The health benefit of whole grain consumption has been attributed to the content of phytochemicals uniquely distributed in the free, soluble-conjugated, and insoluble-bound fractions (*3, 15*). Phytochemicals found in the insoluble-bound fraction, specifically ferulic acid, may survive digestion in the upper gastrointestinal tract and be released by microfloral esterases in the lower gastrointestinal tract (*39*). The release of the compounds in the lower gastrointestinal tract may be responsible for the
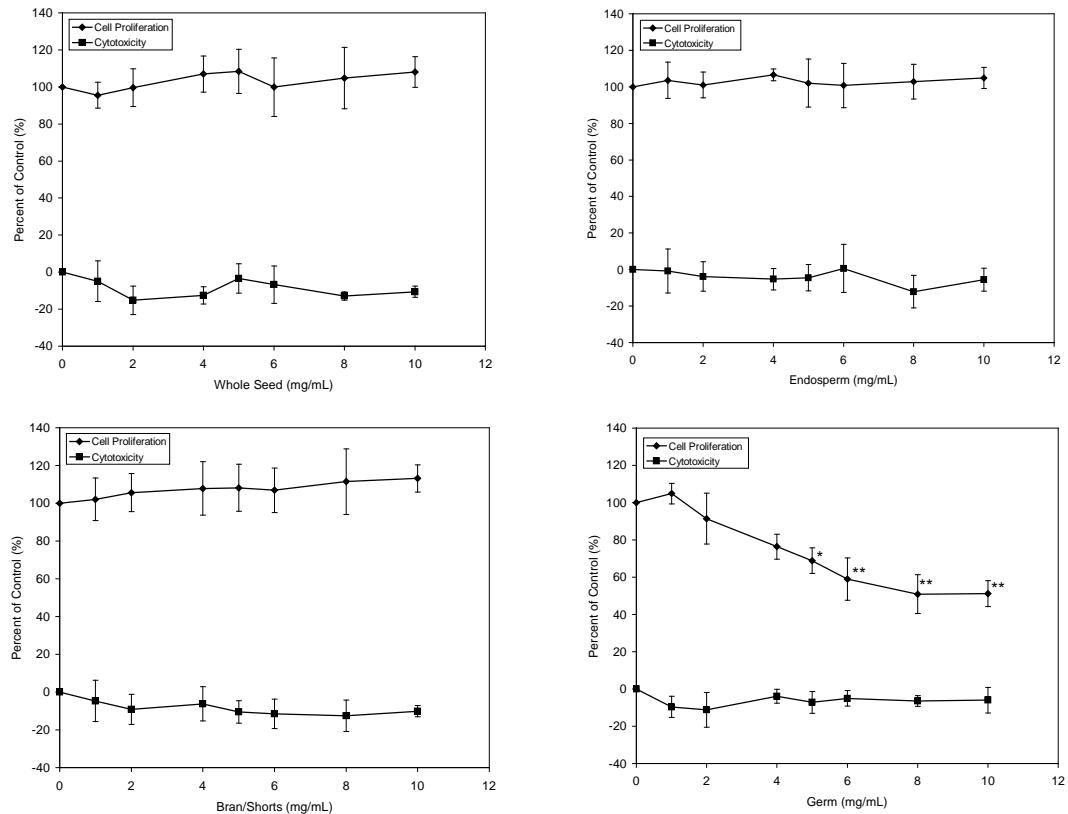
Figure 3.2 – Effects of free phenolics extracts from whole wheat and its milled fractions on cell proliferation in HepG2. Values expressed as percent medium control (mean ± standard deviation, n = 3). * and ** indicate significant differences from medium control at $p < 0.05$ and $p < 0.01$, respectively.
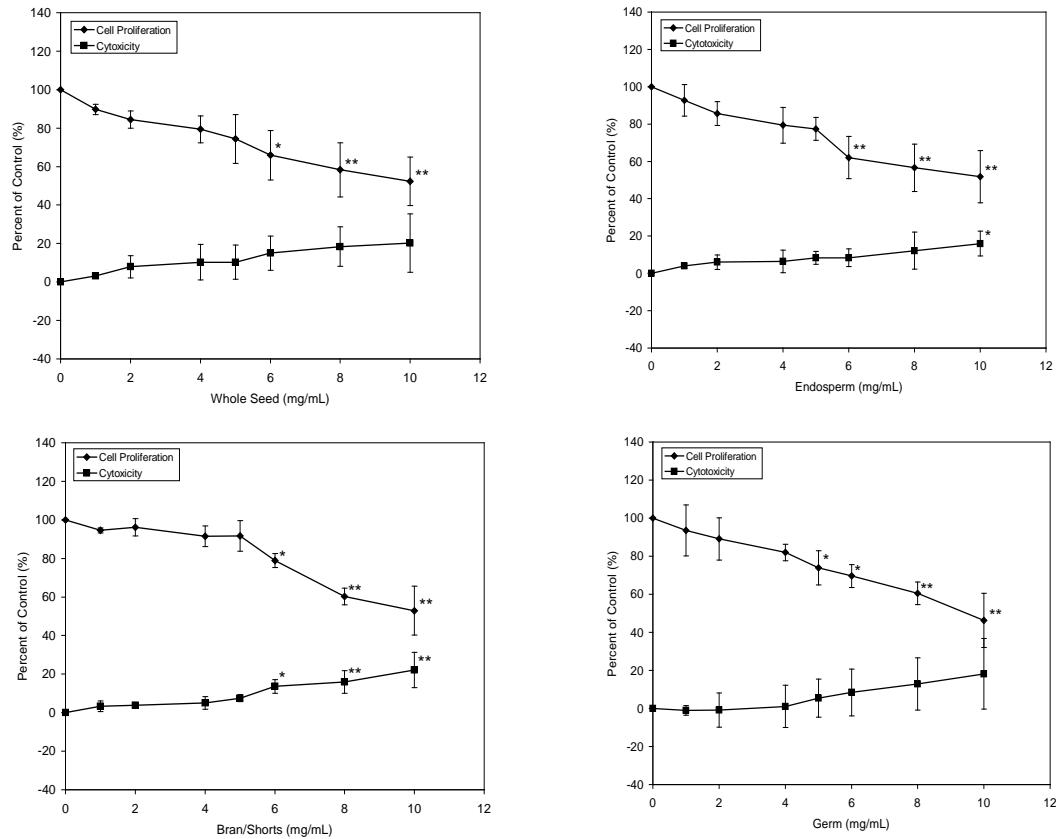
Figure 3.3 – Effects of insoluble-bound phenolics extracts from whole wheat and its milled fractions on cell proliferation in HepG2. Values expressed as percent medium control (mean ± standard deviation, n = 3). * and ** indicate significant differences from medium control at p < 0.05 and p < 0.01, respectively.

association between increased whole grain consumption and reduced risk of colon cancer (*15*).

3.4.2 Total Phenolic Content and Phenolic Composition of Whole Wheat and its Milled Fractions

The total phenolic content and phenolic composition of whole wheat and its milled fractions has been previously reported in the literature (*16, 21, 40-44*). The total phenolic contents and phenolic acid contents reported in the present study are in general agreement with previously reported values. Ferulic acid was the predominant phenolic acid found in whole wheat and its milled fractions. *p*-Coumaric acid, syringic acid, and caffeic acid were also found in whole wheat and its milled fractions, though the endosperm did not contain any syringic acid or caffeic acid. To our knowledge, the free, soluble-conjugated, and insoluble-bound total phenolic content and phenolic acid content of the germ fraction of wheat has not been reported. This is the first study to report the free and insoluble-bound total phenolic content and the free, soluble-conjugated, and insoluble-bound phenolic acid content of the germ. Asenstorfer *et al* reported the presence of apigenin-C-diglycosides in the germ fraction of whole wheat and suggested that the presence of flavonoids in refined wheat flour may be due redistribution of phytochemicals during the milling process (*45*). Further, the authors provided no data with regard to what fraction the apigenin-C-diglycosides came from. In the present study, no flavonoids (quercetin, kaempferol, or catechin) were detected in the free, soluble-conjugated, or insoluble-bound fraction of whole wheat or any of its milled fractions.

4.3 Carotenoid and Vitamin E Content of Whole Wheat and its Milled Fractions

The carotenoid and vitamin E content from whole wheat and its milled fractions has been reported previously in the literature (*16, 21, 42, 44, 46-48*). The carotenoid and vitamin E contents reported in the present study are in general agreement with values previously reported in literature. Mainly α- and β- forms of vitamin E were detected in whole wheat and its milled fractions, though γ-tocopherol was reported in the germ fraction (**Table 3.3**). β-Tocotrienol was the predominant form of vitamin E found in whole wheat and the endosperm. The germ fraction contained mainly tocopherols, though the bran fraction contained mainly tocotrienols. However, the vitamin E contents reported in the present study are lower than those reported by Hidalgo *et al* (2006). Lutein was the predominant carotenoid detected in the present study. Other carotenoids (β-cryptoxanthin, zeaxanthin, and β-carotene) were also detected. No α-carotene was detected in whole wheat or any of its milled fractions.

3.4.4 Antioxidant Activity of Whole Wheat and its Milled Fractions

Previous studies have reported the total phenolic content and ORAC of whole wheat and its milled fractions (*21, 42, 49*). This study reports the total phenolic content and ORAC of both the free and insoluble-bound fractions of whole wheat and its milled fractions. The ORAC of each sample correlated with the total phenolic content. This is consistent with previous findings (*21, 50*). Mateo Anson *et al* reported that there was a strong correlation ($p < 0.0001$) between ferulic acid content in the aleurone layer of the bran and antioxidant capacity as measured by the Trolox Equivalent Antioxidant Capacity (TEAC) Assay (*51*). The strong correlation may be

due to the finding that ferulic acid is the predominant phenolic acid found in the bran fraction of whole wheat (**Table 3.1**).

3.4.5 Grain Extracts and Antiproliferative Activity

The antiproliferative activity of extracts from whole grains and their milled fractions has rarely been reported in the literature. Extracts from brown rice and brown rice bran, consisting mainly of phenolic acids, were able to inhibit the proliferation of three breast and three colon cancer cell lines (*52*). In the present study, free phenolics extracts from the germ were able to significantly inhibit ($p < 0.01$) the proliferation of HepG2 cells after 96 hours. However, free phenolics extracts from the bran/shorts, endosperm, and whole seed had no inhibitory effect on cell proliferation in HepG2 cells. This may be due to the phenolic acid content and distribution between the free, soluble-conjugated and insoluble-bound fractions. The free fraction of the germ contains 68% and 94% of the total ferulic acid and *p*-coumaric acid content of the germ, respectively. However, the free fraction of the bran/shorts contains 15% and 47% of the total ferulic acid and *p*-coumaric acid content of the bran/shorts, respectively. Further, the germ is the only milled fraction that contained caffeic acid in the free fraction.

Insoluble-bound phenolics extracts from whole wheat and its milled fractions were able to significantly ($p < 0.01$) inhibit the proliferation of HepG2 cells at a concentration of 10 mg/mL after 96 hours. However, the total insoluble-bound phenolic content was not significantly ($p > 0.05$) correlated with antiproliferative activity in HepG2 after 96 hours. Liyana-Pathirana and Shahidi (2006) discussed the importance of the insoluble-bound fraction of whole wheat due to its higher total phenolic content and antioxidant activity. This study shows that the insoluble-bound

fraction of whole wheat and its milled fractions is also important because of its significant (p < 0.01) antiproliferative activity in HepG2 cell cultures.

3.4.6 Percent Contribution of each Milled Fraction to the Total

The percent contribution of each milled fraction to the total phenolic content, ferulic acid and *p*-coumaric acid content, tocopherol and tocotrienol content, carotenoid content, and ORAC is summarized in **Figure 3.4**. The percent contribution of each milled fraction to the total phenolic content and ORAC is similar. This is reflective of the significant correlation between total phenolic content and antioxidant activity previously reported in the literature (*15, 21, 42, 50*) and observed in this study. The bran/shorts and germ fractions, together, contribute the most to the total ferulic acid and *p*-coumaric acid contents of the whole seed, though the endosperm contributes more to each than the germ fraction. The percent contribution of each milled fraction to individual carotenoid content was similar with the exception of zeaxanthin. The bran/shorts fraction contributed 51% to the reconstituted whole seed content of zeaxanthin, though it contributed roughly 20% to the reconstituted whole seed content of lutein, β-carotene, and β-cryptoxanthin (**Figure 3.4**). The germ fraction contributed 30% to the reconstituted whole seed content of zeaxanthin, though it contributed roughly 6% to the reconstituted whole seed content of lutein, β-carotene, and β-cryptoxanthin. The endosperm contributed 19% to the reconstituted whole seed content of zeaxanthin, though it contributed roughly 74% to the reconstituted whole seed content of lutein, β-carotene, and β-cryptoxanthin. The percent contribution of each milled fraction to the total tocotrienol content varied greatly. The bran/shorts fraction contributed 67.1% to the total tocotrienol content followed by the endosperm and germ with 31.6 and 1.4%, respectively. Though the bran/shorts fraction contributed the most to the total tocotrienol content, the endosperm contributed
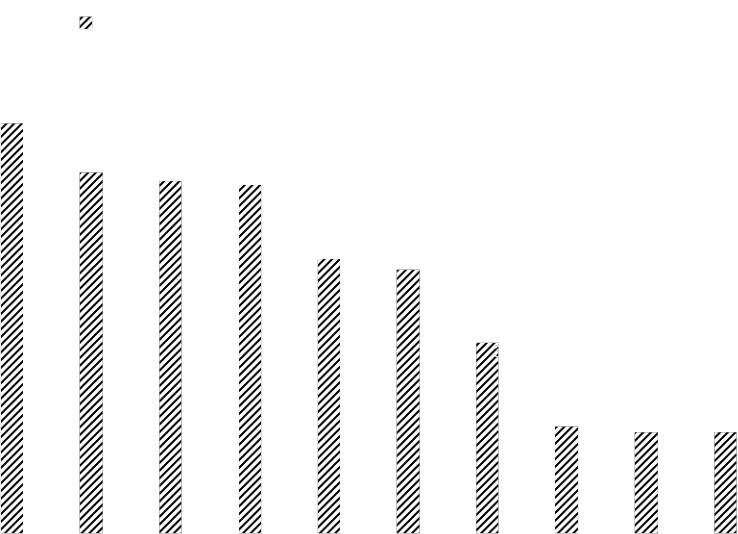
Figure 3.4 – Percent contribution of each milled fraction to the phytochemical content and ORAC of the whole seed.

roughly the same percentage of tocotrienols and tocopherols to the total tocotrienol and total tocopherol content of the whole seed.

3.4.7 Potential Use of the Germ as a Functional Ingredient

The reduction in total phenolic content, phenolic acid content, and vitamin E content upon the milling of whole wheat to refined wheat generates the need for functional components to be added back to refined wheat in order to regain the potential benefit of whole wheat consumption. The germ fraction has the highest total phenolic content, total ferulic acid content, total *p*-coumaric acid content, total carotenoid content and tocopherol content. Further, free and insoluble-bound phenolics extracts from the germ have inhibitory effects on cell proliferation in HepG2 cells. These findings suggest that the germ fraction may be a good candidate for a functional ingredient in wheat-based food products due to its high total phenolic content, high phenolic acid content, high tocopherol content, high antioxidant activity, and ability of phenolic extracts from the free and insoluble-bound fractions to inhibit the proliferation of HepG2 cell cultures after 96 hours. Hemery discussed various dry processing methods that could be used in order to enhance the nutritional quality of wheat products (*53*). Leenhardt *et al* suggested that the germ fraction of the wheat seed may be a good candidate for a functional ingredient in wheat food products due to the low losses of vitamin E during various processing steps in breadmaking, though the losses found for carotenoids were much larger (*54*). Processes to recover the germ fraction after milling have been reported previously (*2*) and in the present study. After recovery of the germ after milling, the germ would need to be preserved and protected from oxidation, as to maintain any possible health benefit that may be imparted by the germ fraction. The effect of processing on the total phenolic content, phenolic acid content and distribution, antioxidant activity, and antiproliferative activity of whole

115

wheat flour containing the germ fraction as a functional ingredient would need to be determined. Further, the effect of adding the germ fraction to the whole flour on the various sensory aspects of the functional food product would need to be investigated. In conclusion, the germ fraction of whole wheat is a good candidate for a functional food ingredient in wheat-based food products.

LITERATURE CITED

1.   AACC *AACC Members Agree on Definition of Whole Grain*; AACC St. Paul, MN 1999.

2.   Posner, E. S.; Li, Y. Z., A Technique for Separation of Wheat Germ by Impacting and Subsequent Grinding *Journal of Cereal Science* **1991,** 13, 49-70.

3.   Liu, R. H., Whole grain phytochemicals and health. *Journal of Cereal Science* **2007,** 46, (3), 207-219.

4.   Okarter, N.; Liu, R. H., Health Benefits of Whole Grain Phytochemicals. . *Critical Reviews in Food Science and Nutrition* **2009,** (in press)

5.   Liu, R. H., Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J Nutr* **2004,** 134, (12 Suppl), 3479S-3485S.

6.   Distaam, E. M.; Carcea, M., Pasta from nontraditional raw materials *Cereal Foods World* **2001,** 46, 522-530.

7.   Seib, P. A.; Liang, X.; Guan, F.; Liang, Y. T.; Yang, H. C., Comparison of Asian noodles from some hard white and hard red wheat flours. *Cereal Chemistry* **2000,** 77, (6), 816-822.

8.   Adom, K. K.; Sorrells, M. E.; Liu, R. H., Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. *J Agric Food Chem* **2005,** 53, (6), 2297-306.

9.   Slavin, J. L.; Jacobs, D.; Marquart, L., Grain processing and nutrition. *Crit Rev Biotechnol* **2001,** 21, (1), 49-66.

10.   Masud, T.; Mahmood, T.; Latif, A.; Sammi, S.; Hameed, T., Influence of Processing and Cooking Methodologies for Reduction of Phytic Acid Content in Wheat (Triticum aestivum) Varieties *Journal of Food Processing and Preservation* **2007,** 31, (5), 583-594.

11.   Dewettinck, K.; Van Bockstaele, F.; Kuhne, B.; de Walle, D. V.; Courtens, T. M.; Gellynck, X., Nutritional value of bread: Influence of processing, food interaction and consumer perception. *Journal of Cereal Science* **2008,** 48, (2), 243-257.

12.   Liyana-Pathlrana, C. M.; Shahidi, F., Antioxidant and free radical scavenging activities of whole wheat and milling fractions. *Food Chemistry* **2007,** 101, (3), 1151-1157.

13.   Siebenhandl, S.; Grausgruber, H.; Pellegrini, N.; Del Rio, D.; Fogliano, V.; Pernice, R.; Berghofer, E., Phytochemical profile of main antioxidants in different

117

fractions of purple and blue wheat, and black barley. *Journal of Agricultural and Food Chemistry* **2007,** 55, (21), 8541-8547.

14.   AOAC, *AOAC Official Methods of Analysis* 15 ed.; Washington DC, 1990; Vol. 2.

15.   Adom, K. K.; Liu, R. H., Antioxidant activity of grains. *J Agric Food Chem* **2002,** 50, (21), 6182-7.

16.   Adom, K. K.; Sorrells, M. E.; Liu, R. H., Phytochemical profiles and antioxidant activity of wheat varieties. *J Agric Food Chem* **2003,** 51, (26), 7825-34.

17.   Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M., Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In *Oxidants and Antioxidants, Pt A*, 1999; Vol. 299, pp 152-178.

18.   Dewanto, V.; Wu, X.; Liu, R. H., Processed sweet corn has higher antioxidant activity. *J Agric Food Chem* **2002,** 50, (17), 4959-64.

19.   Kaiser, H., Quantitation in Elemental Analysis. *Analytical Chemistry* **1970,** 4, 24A - 59A

20.   Liu, C. S.; Glahn, R. P.; Liu, R. H., Assessment of Carotenoid Bioavailability of Whole Foods Using a Caco-2 Cell Culture Model Coupled with an in Vitro Digestion. *J. Agric. Food Chem.* **2004,** 52, (13), 4330-4337.

21.   Okarter, N.; Liu, C. S.; Sorrells, M. E.; Liu, R. H., Phytochemical content and antioxidant activity of six diverse varieties of whole wheat. . *Journal of Food Chemistry* **2009,** (in press)

22.   Panfili, G.; Fratianni, A.; Irano, M., Normal phase high-performance liquid chromatography method for the determination of tocopherols and tocotrienols in cereals. *J Agric Food Chem* **2003,** 51, (14), 3940-4.

23.   Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Prior, R. L., High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. *J. Agric. Food Chem.* **2002,** 50, (16), 4437-4444.

24.   Wolfe, K. L.; Kang, X.; He, X.; Dong, M.; Zhang, Q.; Liu, R. H., Cellular Antioxidant Activity of Common Fruits. *J. Agric. Food Chem.* **2008,** 56, (18), 8418-8426.

25.   Wolfe, K. L.; Liu, R. H., Cellular Antioxidant Activity (CAA) Assay for Assessing Antioxidants, Foods, and Dietary Supplements. *J Agric Food Chem* **2007**.

26.   Liu, R. H.; Sun, J., Antiproliferative activity of apples is not due to phenolic-induced hydrogen peroxide formation. *Journal of Agricultural and Food Chemistry* **2003,** 51, (6), 1718-1723.

27.   Sun, J.; Chu, Y. F.; Wu, X.; Liu, R. H., Antioxidant and antiproliferative activities of common fruits. *J Agric Food Chem* **2002,** 50, (25), 7449-54.

28.   Felice, D. L.; Sun, J.; Liu, R. H., A modified methylene blue assay for accurate cell counting. *Journal of Functional Foods* **2009,** 1, (1), 109-118.

29.   Yoon, H.; Liu, R. H., Effect of selected phytochemicals and apple extracts on NF-kappaB activation in human breast cancer MCF-7 cells. *J Agric Food Chem* **2007,** 55, (8), 3167-73.

30.   Anderson, J. W., Whole grains and coronary heart disease: the whole kernel of truth. *Am J Clin Nutr* **2004,** 80, (6), 1459-60.

31.   Liu, S.; Stampfer, M. J.; Hu, F. B.; Giovannucci, E.; Rimm, E.; Manson, J. E.; Hennekens, C. H.; Willett, W. C., Whole-grain consumption and risk of coronary heart disease: results from the Nurses' Health Study. *Am J Clin Nutr* **1999,** 70, (3), 412-9.

32.   Lockheart, M. S.; Steffen, L. M.; Rebnord, H. M.; Fimreite, R. L.; Ringstad, J.; Thelle, D. S.; Pedersen, J. I.; Jacobs, D. R., Jr., Dietary patterns, food groups and myocardial infarction: a case-control study. *Br J Nutr* **2007,** 98, (2), 380-7.

33.   Fung, T. T.; Hu, F. B.; Pereira, M. A.; Liu, S.; Stampfer, M. J.; Colditz, G. A.; Willett, W. C., Whole-grain intake and the risk of type 2 diabetes: a prospective study in men. *Am J Clin Nutr* **2002,** 76, (3), 535-540.

34.   Montonen, J.; Knekt, P.; Jarvinen, R.; Aromaa, A.; Reunanen, A., Whole-grain and fiber intake and the incidence of type 2 diabetes. *Am J Clin Nutr* **2003,** 77, (3), 622-629.

35.   Munter, J. S. L. d.; Hu, F. B.; Spiegelman, D.; Franz, M.; Dam, R. M. v., Whole grain, bran, and germ intake and risk of type 2 diabetes: a prospective cohort study and systematic review. *PLoS Medicine* **2007,** 4, (8), e261.

36.   Jacobs, D. R., Jr.; Slavin, J.; Marquart, L., Whole grain intake and cancer: a review of the literature. *Nutr Cancer* **1995,** 24, (3), 221-9.

37.   Larsson, S. C.; Giovannucci, E.; Bergkvist, L.; Wolk, A., Whole grain consumption and risk of colorectal cancer: a population-based cohort of 60 000 women. *British Journal of Cancer* **2005,** 92, (9), 1803-1807.

38.   Schatzkin, A.; Mouw, T.; Park, Y.; Subar, A. F.; Kipnis, V.; Hollenbeck, A.; Leitzmann, M. F.; Thompson, F. E., Dietary fiber and whole-grain consumption in

relation to colorectal cancer in the NIH-AARP Diet and Health Study. *Am J Clin Nutr* **2007,** 85, (5), 1353-60.

39.   Andreasen, M. F.; Kroon, P. A.; Williamson, G.; Garcia-Conesa, M. T., Intestinal release and uptake of phenolic antioxidant diferulic acids. *Free Radic Biol Med* **2001,** 31, (3), 304-14.

40.   Li, L.; Shewry, P. R.; Ward, J. L., Phenolic acids in wheat varieties in the HEALTHGRAIN Diversity Screen. *J Agric Food Chem* **2008,** 56, (21), 9732-9.

41.   Mattila, P.; Pihlava, J.-m.; Hellstrom, J., Contents of Phenolic Acids, Alkyl- and Alkenylresorcinols, and Avenanthramides in Commercial Grain Products. *Journal of Agricultural and Food Chemistry* **2005,** 53, (21), 8290-8295.

42.   Moore, J.; Hao, Z.; Zhou, K.; Luther, M.; Costa, J.; Yu, L. L., Carotenoid, tocopherol, phenolic acid, and antioxidant properties of Maryland-grown soft wheat. *J Agric Food Chem* **2005,** 53, (17), 6649-57.

43.   Zhou, K.; Laux, J. J.; Yu, L., Comparison of Swiss red wheat grain and fractions for their antioxidant properties. *J Agric Food Chem* **2004,** 52, (5), 1118-23.

44.   Zhou, K.; Yin, J. J.; Yu, L. L., Phenolic acid, tocopherol and carotenoid compositions, and antioxidant functions of hard red winter wheat bran. *J Agric Food Chem* **2005,** 53, (10), 3916-22.

45.   Asenstorfer, R. E.; Wang, Y.; Mares, D. J., Chemical structure of flavonoid compounds in wheat (Triticum aestivum L.) flour that contribute to the yellow colour of Asian alkaline noodles. *Journal of Cereal Science* **2006,** 43, (1), 108-119.

46.   Barnes, P. J., Composition of Cereal Germ Preparations *Z Lebensm Unters Forsch* **1982,** 174, 467-471.

47.   Hakansson, B.; Jagerstad, M., The Effect of Thermal Inactivation of Lipoxygenase on the Stability of Vitamin E in Wheat *Journal of Cereal Science* **1990** 12, 177-185

48.   Hidalgo, A.; Brandolini, A.; Pompei, C.; Piscozzi, R., Carotenoids and tocols of einkorn wheat (Triticum monococcum ssp monococcum L.). *Journal of Cereal Science* **2006,** 44, (2), 182-193.

49.   Moore, J.; Cheng, Z.; Su, L.; Yu, L., Effects of Solid-State Enzymatic Treatments on the Antioxidant Properties of Wheat Bran. *J. Agric. Food Chem.* **2006,** 54, (24), 9032-9045.

50.   Liyana-Pathirana, C. M.; Shahidi, F., Importance of Insoluble-Bound Phenolics to Antioxidant Properties of Wheat. *J. Agric. Food Chem.* **2006,** 54, (4), 1256-1264.

51.   Mateo Anson, N.; van den Berg, R.; Havenaar, R.; Bast, A.; R. M. M. Haenen, G., Ferulic Acid from Aleurone Determines the Antioxidant Potency of Wheat Grain (Triticum aestivum L.). *Journal of Agricultural and Food Chemistry* **2008,** 56, (14), 5589-5594.

52.   Hudson, E. A.; Dinh, P. A.; Kokubun, T.; Simmonds, M. S. J.; Gescher, A., Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. *Cancer Epidemiology Biomarkers & Prevention* **2000,** 9, (11), 1163-1170.

53.   Hemery, Y.; Rouau, X.; Lullien-Pellerin, V.; Barron, C.; Abecassis, J., Dry processes to develop wheat fractions and products with enhanced nutritional quality. *Journal of Cereal Science* **2007,** 46, (3), 327-347.

54.   Leenhardt, F.; Lyan, B.; Rock, E.; Boussard, A.; Potus, J.; Chanliaud, E.; Remesy, C., Wheat Lipoxygenase Activity Induces Greater Loss of Carotenoids than Vitamin E during Breadmaking. *J. Agric. Food Chem.* **2006,** 54, (5), 1710-1715.

CHAPTER 4

SUMMARY

Goal of the Study

Americans consume more refined grains than whole grains and are therefore not receiving the potential health benefits of whole grain phytochemicals (*1*). Wheat is consumed in the largest amounts compared to any other grain. Therefore, determining the phytochemical content and antioxidant activity and antiproliferative activity of whole wheat is a good starting point towards understanding the potential health benefit of whole grain consumption. The goal of this study was to determine the phytochemical content and antioxidant activity of six diverse varieties of whole wheat and the phytochemical content, antioxidant activity, and antiproliferative activity of whole wheat and its milled fractions.

Research Findings

I determined the phytochemical content of six diverse varieties of whole wheat. The total phenolic content ranged from 841 (KanQueen) to 1099 (Stoa) μmol gallic acid equivalents/100 g DW. The insoluble-bound fraction contributed 53.8 to 69.7% of the total phenolic content of the wheat varieties analyzed. Ferulic acid was the predominant phenolic acid found in whole wheat. p-Coumaric acid, syringic acid, vanillic acid, and caffeic acid were also detected. Lutein was the predominant carotenoid in the whole wheat varieties analyzed. Zeaxanthin, β-carotene, and β-cryptoxanthin were also detected. Mainly α- and β-tocopherols and -tocotrienols were found in all varieties of whole wheat. γ-Tocopherol was also detected in all but two varieties. β-Tocotrienol was the predominant form of vitamin E in all varieties of whole wheat.

I also determined the phytochemical content of whole wheat and its milled fractions (the germ, bran/shorts, and endosperm fractions). The total phenolic content of the germ, bran/shorts, whole seed, and endosperm were 2140, 2020, 897, and 307 μmol gallic acid equivalents/100 g DW, respectively. Ferulic acid was the predominant phenolic acid found in whole wheat and its milled fractions. p-Coumaric acid, syringic acid, p-hydroxybenzoic acid, and caffeic acid were also detected. No flavonoids (quercetin, kaempferol, and catechin) were detected in whole wheat or any of it milled fractions. Lutein was the predominant carotenoid in whole wheat and its milled fractions. Again, α- and β-tocopherols and -tocotrienols were detected in whole wheat and its milled fractions. γ-Tocopherol was detected in the germ fraction. The bran/shorts fraction consisted mainly of tocotrienols, though the germ fraction consisted mainly of tocopherols. The whole seed contained significantly more tocotrienols than the endosperm, though the tocopherol content of the whole seed and endosperm was similar.

Relevance of the Research

The phenolic compounds found in whole wheat may be able to scavenge free radicals and inhibit the proliferation of cancer cells *in vitro*. I determined the antioxidant activity of six diverse varieties of whole wheat and a commercial blend of whole wheat and its milled fractions was assessed using the ORAC assay. I used a modification of the methylene blue stain assay to determine the antiproliferative activity of phenolics extracts from the free and insoluble-bound fraction of a commercial blend of whole wheat and its milled fraction (bran/shorts, germ, and endosperm). I paid specific attention to the insoluble-bound fraction of each sample because phenolic compounds from insoluble-bound fraction of whole wheat and its milled fractions may survive digestion in the upper gastrointestinal tract and impart

their potential health benefits in the lower gastrointestinal tract. The potential health benefits in the lower gastrointestinal tract may have implications with regards to increased whole grain consumption and reduced risk of colon cancer.

The total antioxidant activity of the six diverse varieties of whole wheat ranged from 5148 (KanQueen) to 9616 (Stoa) μmol Trolox equivalents/100 g DW. The antioxidant activity of the insoluble-bound fraction of the whole wheat samples used in this study ranged from 3190 to 5945 μmol Trolox equivalents/100 g DW and accounted for 52.8-68.8% of the total antioxidant activity. The germ had the highest total antioxidant activity with 18380 μmol TE/100 g DW, followed by the bran/shorts, whole seed, and endosperm with 16487, 7133, and 2583 μmol TE/100 g DW, respectively. The antioxidant activity of the insoluble-bound fraction of the germ and the whole seed were statistically similar ($p > 0.05$). The total antioxidant activity of the bran/shorts and the germ were statistically similar ($p > 0.05$).

The antiproliferative activity of whole wheat and its milled fractions was determined in HepG2 cells using a modification of the methylene blue stain assay. Phenolics extracts from the free fraction of the germ fraction significantly inhibited HepG2 cell proliferation. Free phenolics extracts from the whole seed, bran/shorts, and endosperm had no inhibitory effects on HepG2 cell proliferation. However, phenolics extracts from the insoluble-bound fraction of whole wheat and its milled fractions significantly inhibited HepG2 cell proliferation.

Conclusions

Based on the data I provide in this dissertation, I speculate that any potential health benefit in the lower gastrointestinal tract from whole wheat consumption is due to the ability of phenolics compounds from the insoluble-bound fraction of whole wheat to scavenge free radicals. Further, the potential health benefit of whole wheat is

most likely not due to the antiproliferative activity of these compounds, as phenolics extracts from the insoluble-bound fraction of whole and refined wheat have similar antiproliferative effects. Adding the germ fraction back to the refined wheat after processing may replace any lost potential health benefit by adding back the antioxidant activity that was lost as a result of primary processing.

Future Issues

In this dissertation, I address the ability of phenolics extracts from the insoluble-bound fraction of whole wheat and its milled fractions to scavenge free radicals and inhibit the proliferation of cell cultures *in vitro*. The data in this dissertation suggest that phenolics extracts from the insoluble-bound fraction of whole wheat have a greater capacity to scavenge free radicals than phenolics extracts from the insoluble-bound fraction of refined wheat. However, the antioxidant activity assay used in this dissertation to determine the antioxidant activity of whole wheat does not take into account factors such as uptake and metabolism of the phenolic compounds (*2-7*). Further, this antioxidant activity assay has not been standardized to any other antioxidant and therefore cannot be compared to one another (*8*). The cellular antioxidant activity assay addresses the issues of uptake and metabolism of phenolic compounds under more physiologically relevant conditions (*9*). The cellular antioxidant activity of phenolics extracts from whole grains should be determined.

In Chapter 3 of this dissertation, I report that insoluble-bound fraction of whole wheat and its milled fractions had significant antiproliferative activity in HepG2 cells. These data suggest that both whole wheat and refined wheat may be able to protect against cancers by inhibiting the proliferation of cancer cells. It is important to note that cells derived from an environment that is high in dietary components may be able to tolerate higher concentrations of dietary compounds (*10*). Therefore, it may not be

125

appropriate to investigate the antiproliferative activity of phenolics extracts from the insoluble-bound fraction of whole and refined wheat in HepG2 cells or any other cell line that is not derived from the intestinal epithelium.

It is also important to realize that human beings do not eat whole or refined wheat. Whole wheat and refined wheat flours are further processed into the food products that we eat. Secondary processing methods, baking, boiling, steaming, and kneading may also further reduce the phenolic content and composition of whole and refined wheat flour. Previous studies have reported changes to phenolic content and antioxidant activity of whole grains secondary processing (*11-18*). However, the data with regards to the secondary processing of grains and antioxidant activity are also mixed and hard to compare as different antioxidant activity assays were used in these studies.

It is also important to determine the antiproliferative activity of phenolics extracts from the insoluble-bound fraction of whole and refined wheat after primary processing (milling) and secondary processing. The additional processing steps may remove phenolic compounds to the point where the concentration of phenolic compounds is not high enough to elicit antiproliferative activity.

This dissertation also lays the foundation for future research regarding the cellular antioxidant activity of whole grains and the effects of primary and secondary processing of grains on antioxidant activity and cancer cell proliferation.

LITERATURE CITED

1.   Lin, B. Y.; Yen, S. T. *The U.S. Grain Consumption Landscape: Who eats grain, in what form, where, and how much?* ; United States Department of Agriculture 2007

2.   Adom, K. K.; Liu, R. H., Antioxidant activity of grains. *J Agric Food Chem* **2002,** 50, (21), 6182-7.

3.   Adom, K. K.; Sorrells, M. E.; Liu, R. H., Phytochemical profiles and antioxidant activity of wheat varieties. *J Agric Food Chem* **2003,** 51, (26), 7825-34.

4.   Adom, K. K.; Sorrells, M. E.; Liu, R. H., Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. *J Agric Food Chem* **2005,** 53, (6), 2297-306.

5.   Liyana-Pathirana, C. M.; Shahidi, F., Importance of Insoluble-Bound Phenolics to Antioxidant Properties of Wheat. *J. Agric. Food Chem.* **2006,** 54, (4), 1256-1264.

6.   Moore, J.; Hao, Z.; Zhou, K.; Luther, M.; Costa, J.; Yu, L. L., Carotenoid, tocopherol, phenolic acid, and antioxidant properties of Maryland-grown soft wheat. *J Agric Food Chem* **2005,** 53, (17), 6649-57.

7.   Mpofu, A.; Sapirstein, H. D.; Beta, T., Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. *Journal of Agricultural and Food Chemistry* **2006,** 54, (4), 1265-1270.

8.   Fardet, A.; Rock, E.; Remesy, C., Is the in vitro antioxidant potential of whole-grain cereals and cereal products well reflected in vivo? *Journal of Cereal Science: 48 (2) 258-276* **2008,** 48, (2), 258-276.

9.   Wolfe, K. L.; Liu, R. H., Cellular Antioxidant Activity (CAA) Assay for Assessing Antioxidants, Foods, and Dietary Supplements. *J Agric Food Chem* **2007**.

10.   Janicke, B.; Onning, G.; Oredsson, S. M., Differential effects of ferulic acid and p-coumaric acid on S phase distribution and length of S phase in the human colonic cell line Caco-2. *Journal of Agricultural and Food Chemistry* **2005,** 53, (17), 6658-6665.

11.   Cheng, Z.; Su, L.; Moore, J.; Zhou, K.; Luther, M.; Yin, J. J.; Yu, L. L., Effects of postharvest treatment and heat stress on availability of wheat antioxidants. *J Agric Food Chem* **2006,** 54, (15), 5623-9.

12.   Dewanto, V.; Wu, X.; Liu, R. H., Processed sweet corn has higher antioxidant activity. *J Agric Food Chem* **2002,** 50, (17), 4959-64.

13.   Dewettinck, K.; Van Bockstaele, F.; Kuhne, B.; de Walle, D. V.; Courtens, T. M.; Gellynck, X., Nutritional value of bread: Influence of processing, food interaction and consumer perception. *Journal of Cereal Science* **2008,** 48, (2), 243-257.

14.   Gelinas, P.; McKinnon, C. M., Effect of wheat variety, farming site, and bread-baking on total phenolics. *International Journal of Food Science & Technology* **2006,** 41, (3), 329-332.

15.   Hakansson, B.; Jagerstad, M., The Effect of Thermal Inactivation of Lipoxygenase on the Stability of Vitamin E in Wheat *Journal of Cereal Science* **1990** 12, 177-185

16.   Li, W.; Pickard, M. D.; Beta, T., Effect of thermal processing on antioxidant properties of purple wheat bran. *Food Chemistry* **2007,** 104, (3), 1080-1086.

17.   Randhir, R.; Kwon, Y.-I.; Shetty, K., Effect of thermal processing on phenolics, antioxidant activity and health-relevant functionality of select grain sprouts and seedlings. *Innovative Food Science & Emerging Technologies* **2008,** 9, (3), 355-364.

18.   Winata, A.; Lorenz, K., Effects of fermentation and baking of whole wheat and whole rye sourdough breads on cereal alkylresorcinols. *Cereal Chemistry* **1997,** 74, (3), 284-287.

APPENDIX A

PHENOLIC COMPOUNDS FROM THE INSOLUBLE-BOUND FRACTION OF WHOLE GRAINS DO NOT HAVE ANY CELLULAR ANTIOXIDANT ACTIVITY IN HEPG2 CELLS

Abstract

The present study reports the total phenolic content, phenolic composition, and antioxidant activity of eight grains (five cereals and three pseudocereals). Further, the cellular antioxidant activity of four phenolic acids previously reported in whole grains was assessed. Total phenolic content ranged from 71 (amaranth) to 405 (corn) mg GAE/100 g. Ferulic acid and p-coumaric acid were found in all grain samples analyzed though p-coumaric acid was found only in the insoluble-bound fraction, with the exception of corn and oats. Caffeic acid was only detected in the insoluble-bound fraction of barley and corn. No p-hydroxybenzoic acid was detected in the cereals or in the insoluble-bound fraction of buckwheat. Quercetin and rutin were only detected in the free fraction of quinoa and buckwheat and not in the insoluble-bound fraction of any grain. The total ORAC ranged from 2278 (amaranth) to 14273 (corn) μmol TE/100 g grain and was correlated with total phenolic content ($R^2 = 0.880$; $p < 0.001$). None of the four phenolic acids had any cellular antioxidant activity. Of the phenolic acids reported in whole grains, only caffeic acid had any cellular antioxidant activity. Further, the cellular antioxidant activity of caffeic acid was relatively low compared to other flavonoids, particularly quercetin. The phenolic acids found in whole grains lack the structure necessary to impart cellular antioxidant activity.

A.1 Introduction

A whole grain consists of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components - the starchy endosperm, germ and bran - are present in the same relative proportions as they exist in the intact caryopsis (*1*). A list of whole grains was provided in Chapter 1 of this dissertation (*2*). The health benefits of whole grain consumption with regards to cardiovascular disease, type II diabetes, major weight gain, and obesity were also previously reviewed (*3*). Pseudocereals are plants that are not grasses but produce seeds that can be milled and used much the same way as cereal flours (*4*). These pseudocereals do not contain the protein gluten, intolerance to which is known as Celiac disease. Pseudocereals include but are not limited to buckwheat, quinoa, and amaranth.

Phytochemicals are bioactive non-nutrient plant compounds in fruits, vegetables, grains, and other plant foods that have been linked to reduced risk of major chronic diseases (*5*). A major class of phytochemicals is phenolics, compounds containing one or more aromatic ring and one or more hydroxyl group. These compounds include phenolic acids and flavonoids (**Figure A.1**). Phenolic compounds possess antioxidant activity by transferring a hydrogen atom to a free radical and delocalizing the lone electron throughout the aromatic ring.

The multistage model for carcinogenesis involves the initiation of a normal cell, the promotion of an initiated cell to a preneoplasia, and finally the progression of the preneoplasia to an invasive tumor. The initiation of a normal cell can be caused by reactive oxygen species. Reactive oxygen species oxidize biologically important molecules such as DNA. Antioxidant activity assays are used to determine the ability of a compound or an extract to prevent the initiation of a normal cell. The transfer of a hydrogen atom from a phenolic compound to the reactive oxygen species prevents the oxidation of biologically important molecules. After transfer of the hydrogen atom to
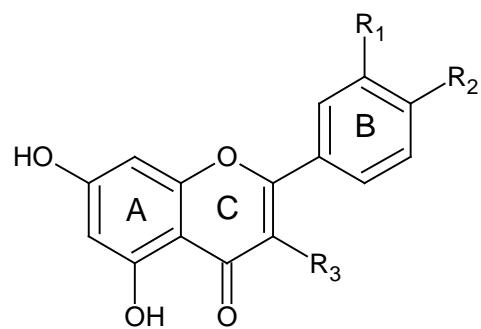
	R_1	R_2	R_3
Quercetin	OH	OH	OH
Kaempferol	H	OH	OH
Rutin	OH	OH	Glu-Glu
Apigenin	OH	H	H

Figure A.1 – Structure of flavonoids

the reactive oxygen species, the antioxidant remains a stable compound by delocalizing the unpaired electron amongst the alternating single and double bonds. Generally, chemistry assays have been used to determine the antioxidant activity of grains (**Table A.1**) (*6-8*). These antioxidant activity assays involve the use of a free radical generator, a probe, and a standard.

Adom and Liu (2002) determined the antioxidant activity of whole corn, wheat, oats, and rice using the TOSC assay (*9*). Zhou *et al* (2004) determined the antioxidant activities of Swiss red wheat and its bran fraction, using the Oxygen Radical Absorbance Capacity (ORAC) assay (*10*). Moore *et al* (2005) determined the antioxidant activity of Maryland-grown soft wheat using the ORAC assay (*11*). Adom and Liu (2005) determined the antioxidant activity of grains using the Peroxyradical Scavenging Capacity (PSC) assay (*6*).

Fardet *et al* questioned whether or not these chemical antioxidant activity assays are well reflected *in vivo* as the previous chemistry assays lack the ability to predict *in vivo* antioxidant activity due to the use of non-physiological temperature and/or pH, no accounting for bioavailability, uptake, or metabolism (*12, 13*).

Wolfe and Liu (2007) developed the Cellular Antioxidant Activity (CAA) assay, in which 2',7'-dichlorofluorosceine diacetate (DCFH-DA) is cleaved by cellular esterases and is used to measure the loss of fluorescence upon quenching of the ABAP-induced reactive oxygen species by the pure compound or sample extract using HepG2 cell cultures (*13*). This assay has been used to determine the cellular antioxidant activity of a wide range of flavonoids and fruits (*14, 15*).

Until present, no studies have investigated the cellular antioxidant activity of grains or the phenolic compounds found in grains aside from ferulic acid and caffeic acid (*13*). The use of a cellular antioxidant activity assay to determine the antioxidant activity of whole grain phenolic compounds may be a more valuable tool than the

132

Table A.1 – List of antioxidant activity assays.

Assay	Initiator	Probe	Standard
Total Oxyradical Scavenging Capacity (TOSC)	ABAP	KMBA	Vitamin C
Oxygen Radical Absorbance Capacity (ORAC)	ABAP	Fluorescein	Trolox
Peroxyl Radical Scavenging Capacity (PSC)	ABAP	DCFH	Vitamin C

ABAP, 2,2'-Azobis (2-amidinopropane) dihydrochloride; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; DCFH, dichlorofluorescein; KMBA, α-keto-γ-methiolbutyric acid.

more commonly used antioxidant activity assays in understanding the potential health benefit of whole grain consumption. The objectives of this study were to 1) determine the total phenolic content, phenolic composition, and oxygen radical absorbance capacity of eight whole grains, and 2) determine the cellular antioxidant activity of the phenolic compounds found in whole grains.

A.2 Materials and Methods

A.2.1 Chemicals and Reagents

Sodium hydroxide, sodium sulfate, and hexanes were purchased from Fisher Scientific (Pittsburgh, PA). Acetone, sodium carbonate, sodium chloride, ethanol, and ethyl acetate were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Folin-Ciocalteu reagent, ferulic acid, p-coumaric acid, caffeic acid, p-hydroxybenzoic acid, vannilic acid, syringic acid, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), were purchased from Sigma (St. Louis, MO). Gallic acid was purchased from ICN Biomedicals, Inc. (Aurora, OH). 2,2'-Azobis (2-amidinopropane) dihydrochloride (ABAP) was purchased from Wako Chemicals USA, Inc. (Richmond, VA). Methylene blue was purchased from BBL (Cockeysville, MD). HepG2 cell cultures were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). Williams' Medium E (WME) and Hanks' Balanced Salt Solution (HBSS) were purchased from Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA).

A.2.2 Grain Samples and Sample Preparation

Grains samples were purchased at a local supermarket in Ithaca, NY. Each sample was milled to a fine powder using 20, 40, and 60 mesh size screens successively, mixed thoroughly and stored at -20˚C.

A.2.3 Extraction of Free Phenolics

Free phenolic compounds were extracted according to the method described by Okarter *et al* (*16*). One gram of grain sample flour was blended with 50 mL of 80% chilled acetone. The mixture was then centrifuged at 2500 G for 10 minutes. The supernatant was removed and the remaining pellet was again extracted with 50 mL of 80% chilled acetone. The supernatants were pooled and evaporated at 45ºC to dryness. The solution was then reconstituted with methanol:hydrochloric acid (1 M, 85:15 v/v) and stored at -40ºC.

A.2.4 Extraction of Insoluble-Bound Phenolics

Insoluble-bound phenolic compounds were extracted from the residue of the free phenolic extraction according to the method described by Okarter *et al* (*16*). The residue was first digested with 2 M sodium hydroxide at room temperature for 1 h whilst shaking under nitrogen. The mixture was then neutralized to pH 2 with an appropriate amount of hydrochloric acid. Hexanes were used to extract lipids in the mixture. The remaining mixture was then extracted with ethyl acetate five times. The ethyl acetate fractions were pooled and evaporated to dryness. The phenolics were reconstituted in methanol:hydrochloric acid (1 M, 85:15 v/v) and stored at -40ºC.

A.2.5 Extraction of Soluble-Conjugated Phenolics

Soluble-conjugated phenolic acids were extracted from free phenolic extracts. The extract was digested with 2 M sodium hydroxide for 1 h at room temperature under nitrogen gas, and then neutralized with concentrated hydrochloric acid. The mixture was then extracted five times with ethyl acetate. The ethyl acetate fractions were pooled and evaporated to dryness under a gentle stream of nitrogen at 35°C. The remaining residue was dissolved in methanol:hydrochloric acid (1 M, 85:15 v/v) and stored at -40°C.

A.2.6 Determination of Total Phenolic Content

Total phenolic content was determined using the colorimetric method described by Singleton *et al* (*17*) modified in our lab (*16*). Briefly, extracts were reacted with Folin-Ciocalteu reagent and then neutralized with sodium carbonate. After 90 minutes, the absorbance of the resulting solution was measured at 760 nm. Gallic acid was used as the standard and total phenolic content was expressed as mg gallic acid equivalents/100 g sample.

A.2.7 Determination of Phenolic Composition

The determination of the phenolic composition was done using the rp-HPLC-DAD method described in Chapter 3 of this dissertation (*2*). Briefly, the mobile phase [water at pH 2.8 with acetic acid (A) and acetonitrile/water 70:30 v/v to pH 2.8 with acetic acid (B)] was delivered using a Waters 600E quaternary pump at a flow of 1.5 mL/min using the following gradient: 0 – 10% B for 2.5 minutes, 10 – 12% B for 2.5 minutes, 12 – 23% B for 10 minutes, 23 – 95% B for 4 minutes, and 95 – 0% B for 4 minutes. Total run time for each injection was 25 minutes. Seventy five µL of sample were injected using a Water 717 Autosampler. Seperation of phenolic

compounds was done using a C18 column (5 μm, 250 mm x 4.6 mm column; Grace Vydac, Baltimore, MD). Phenolic compounds were detected using a Waters 996 Photodiode Array Detector. Each injection was monitored at 315 nm. p-Hydroxybenzoic acid and vanillic acid were quantified at 254 nm. Catechin and syringic acid were quantified at 275 nm. Ferulic acid, p-coumaric acid, salicylic acid, and caffeic acid were quantified at 315 nm. Quercetin, rutin, and kaempferol were quantified at 365 nm. Data signals were acquired and processed using Waters Empower software (2002) (Waters Corp., Milford, MA).

A.2.8 Oxygen Radical Absorbance Capacity

The antioxidant activity was determined using the ORAC assay described by Huang *et al* (*18*) and modified in our laboratory (*2, 16*). Phenolic extract dilutions were prepared with 75 mM phosphate buffer (pH 7.4). The assay was performed in black-walled 96-well plates (Corning Scientific, Corning, NY). The outside wells of the plate were not used as there was much more variation from them than from the inner wells. Each well contained 20 μL extracts or 20 μL Trolox standard (range 6.25 – 50 μM), and 200 μL fluoroscein (final concentration 0.96 μM), which were incubated at 37 °C for 20 minutes. After incubation, 20 μL of 119 mM ABAP was added to each well. Fluorescence intensity was measured using Fluoroskan Ascent FL plate-reader (Thermo Labsystems, Franklin, MA) at excitation of 485 nm and emission of 520 nm for 35 cycles every 5 min. ORAC was expressed as μmol Trolox equivalents/100 g.

A.2.9 Cell Culture

HepG2 cells were grown in William's Medium E (WME) containing 5% FBS, 10 mM HEPES, 2 mM L-glutamine, 5 μg/mL insulin, 0.05 μg/mL hydrocortisone, 50

units/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL gentamicin. Cells were maintained at 37 °C and 5% CO_2. Cells used in this study were between 10 and 25 passages.

A.2.10 Cell Counting Assay

Cell number was determined using a method described by Oliver *et al* (*19*) and modified in our laboratory (*20*). HepG2 cells were seeded at 4 x 10^4 cells per well on a 96-well plate in 100 μL growth medium and incubated for 4 hours at 37°C. The medium was removed and the cells were treated with varying concentrations of p-coumaric acid, vanillic acid, p-hydroxybenzoic acid, or syringic acid prepared in 2% DMSO in growth medium. The plates were incubated at 37°C for 24 h. After 24 h, the treatment medium was removed and the cells were washed with PBS. A volume of 50 μL/well methylene blue fixing/staining solution (98% HBSS, 0.67% glutaraldehyde, 0.6% methylene blue) was applied to each well and the plate was incubated at 37°C for 1 h. The dye was removed and the plates were immersed in fresh deionized water three times, or until the water was clear. The water was tapped out of the wells and the plate was allowed to air-dry before 100 μL elution solution (49% PBS, 50% ethanol, 1% acetic acid) was added to each well. The microplate was placed on a bench-top shaker for 20 minutes to allow uniform elution. The absorbance was read at 570 nm with blank subtraction using the Dynex Technologies MRX II spectrophotometer (Dynex Technologies Inc., Chantilly, VA).

A.2.11 Cellular Antioxidant Activity Assay

The cellular antioxidant activity of each whole wheat sample was determined using the method described by Wolfe and Liu (*13*). Briefly, HepG2 cells were seeded at a density of 6 x 10^4 cells/well on a 96-well microplate in 100 μL growth

medium/well. Twenty-four hours after seeding, the growth medium was removed. Triplicate wells were treated for 1 h with 100 µL treatment medium containing various concentrations of pure phenolic compound plus 25 µM DCFH-DA. Then, 600 µM ABAP was applied to the cells in 100 µL Hank's Balanced Salt Solution (HBSS). Emission at 538 nm was measured with excitation at 485 nm every 5 min for 1 hour with a Fluoroskan Ascent FL plate-reader (ThermoLabsystems, Franklin, MA) at 37°C. Each plate included triplicate control and blank wells: control wells contained cells treated with DCFH-DA and oxidant; blank wells contained cells treated with DCFH-DA and HBSS without oxidant.

A.2.12 Statistical Analysis

Statistical analysis using one-way ANOVA and Tukey's comparison test was performed using Minitab Statistical Software release 15 (Minitab Inc., State College, PA).

A.3 Results

A.3.1 Total Phenolic Content

The total phenolic content of eight grains was determined using the Folic-Ciocalteu Reagent method described by Singleton *et al* (*17*) and modified by our lab (*16*). The phenolic content was expressed as mg gallic acid equivalents/100 g grain (**Figure A.2**). Free phenolic content ranged from 47 (amaranth) to 341 (buckwheat) mg GAE/100 g. Bound phenolic content ranged from 24 (amaranth and buckwheat) to 255 (corn) mg GAE/100 g. Total phenolic content ranged from 71 (amaranth) to 405 (corn) mg GAE/100 g. Generally, the pseudo-cereals contained more free phenolics
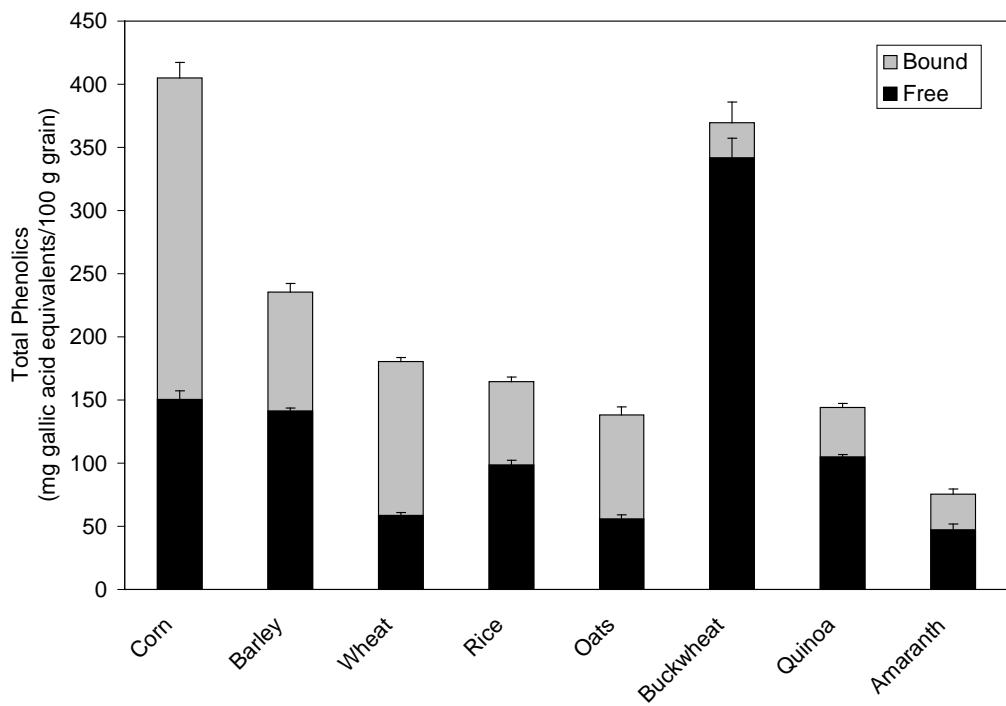
Figure A.2 – Total phenolic content of grains (mean ±SD, n = 3).

than bound and had similar (p > 0.05) bound phenolic contents (24 to 39 mg GAE/100 g).

A.3.2 Phenolic Composition of Grains

Phenolic compounds (phenolic acids and flavonoids) were detected and quantified using a previously described rp-HPLC-DAD assay (*2*). Ferulic acid and *p*-coumaric acid were found in all grain samples analyzed though *p*-coumaric acid was found only in the insoluble-bound fraction, with the exception of corn and oats. No *p*-coumaric acid was detected in the free or soluble-conjugated fractions of buckwheat. Caffeic acid was detected in the insoluble-bound fraction of barley (4.2 ± 1.6 µmol/100 g) and corn (1.8 ± 0.03 µmol/100 g). No p-hydroxybenzoic acid was detected in the cereals. No p-hydroxybenzoic acid was detected in the insoluble-bound fraction of buckwheat. Vanillic acid was detected in quinoa, amaranth, and wheat. Vanillic acid was also detected in the free, soluble-conjugated, and insoluble bound fractions of quinoa but only in the soluble-conjugated fraction of amaranth and wheat. Rutin and quercetin were detected in buckwheat and quinoa. Rutin was detected in the free and soluble-conjugated fractions of quinoa but only in the free fraction of buckwheat. No flavonoids were detected in the cereals or amaranth. No flavonoids were detected in the insoluble-bound fraction of buckwheat or quinoa.

Corn had the most ferulic acid of the cereals (591.3 µmol/100 g) while oats had the least (85.2 µmol/100 g) (**Table A.2**). Qunioa had the most ferulic acid of the pseudo-cereals (62.2 µmol/100 g) while buckwheat had the least (11.5 µmol/100 g). The bound fraction contributed between 78.9% and 94.5% to the total ferulic acid content of the cereals and between 46.0% and 60.0% to the total ferulic acid content of the pseudo-cereals.

The total p-coumaric acid content of the cereals ranged from 18.1 (wheat) to 116.8 (corn) μmol/100 g (**Table A.2**). The total p-coumaric acid content of the pseudo-cereals ranged from 6.3 (buckwheat) to 18.7 (quinoa) μmol/100 g. The insoluble-bound fraction contributed between 60.1 (corn) to 81.5% (rice) to the total p-coumaric acid content of the cereals and between 51.9 (amaranth) to 100% (buckwheat) of the pseudo-cereals.

Hydroxybenzoic acid-derivatives were found almost exclusively in the pseudo-cereals. The total p-hydroxybenzoic acid content of pseudo-cereals ranged from 11.5 (buckwheat) to 39.4 (amaranth) μmol/100 g (**Table A.3**). Most of the total p-hydroxybenzoic acid content came from the free fraction. The total free p-hydroxybenzoic acid content ranged from 11.5 (buckwheat) to 28.2 (amaranth) μmol/100 g. The total free content contributed between 50 (quinoa) to 100% (buckwheat) to the total p-hydroxybenzoic acid content. The insoluble-bound p-hydroxybenzoic acid content of amaranth and quinoa was 11.2 and 15.2 μmol/100 g, respectively.

The total vanillic acid content ranged from 13.9 (amaranth) to 52.5 (quinoa) μmol/100 g (**Table A.3**). The total vanillic acid content of wheat was 15.5 μmol/100 g. The total free vanillic acid content of quinoa contributed 65.0% to the total vanillic acid content.

The total rutin content of buckwheat and quinoa was 18.2 and 75.3 μmol/100 g, respectively (**Table A.4**). The free and soluble-conjugated rutin contents of quinoa were 71.5 and 3.8 μmol/100 g, respectively. Quercetin was detected in the free fraction of quinoa. The total quercetin content of quinoa was 3.7 μmol/100 g.

Table A.2 – Hydroxycinnamic acid-derivative content of grains. Values reported as μmol/100 g (mean ± SD, n = 3). Values with no letter in common within each column are significantly different (p < 0.05). Percent contribution to the whole seed is in parentheses. nd – not detected

	Free	Soluble-Conjugated	Total Soluble[a]	Insoluble-Bound	Total[b]
			Ferulic Acid		
Corn	10.5 ± 4.0 b (1.8%)	22.4 ± 2.4 (3.8%)	32.8 ± 6.4 ab (5.5%)	558 ± 8.8 a (94.5%)	591 ± 2.8 a
Wheat	9.5 ± 3.7 b (4.1%)	27.7 ± 21.4 (12.1%)	37.2 ± 18.5 a (15.8%)	192 ± 15.7 b (83.6%)	229 ± 30.0 b
Barley	4.9 ± 0.03 b (3.8%)	16.7 ± 0.9 (10.8%)	21.6 ± 0.9 ab (14.0%)	133 ± 12.2 c (86.0%)	155 ± 11.4 c
Rice	6.9 ± 0.3 b (6.1%)	16.8 ± 3.0 (15.0%)	23.7 ± 2.7 ab (21.1%)	88.6 ± 6.5 d (78.9%)	112 ± 6.7 d
Oats	5.7 ± 0.6 b (6.7%)	9.3 ± 0.6 (10.9%)	15.0 ± 1.2 ab (17.7%)	70.2 ± 7.4 d (82.4%)	85.2 ± 7.1 d
Quinoa	26.7 ± 18.0 a (40.0%)	nd	26.7 ± 18.0 ab (40.0%)	35.5 ± 5.1 e (60.0%)	62.2 ± 23.1 d
Buckwheat	nd	6.3 ± 1.0 (54.1%)	6.3 ± 1.0 (54.1%)	5.3 ± 0.2 f (46.0%)	11.5 ± 0.8 e
Amaranth	nd	6.5 ± 0.6 (21.7%)	6.5 ± 0.6 (21.7%)	23.3 ± 1.7 e (78.5%)	29.7 ± 2.1 e
			p-Coumaric Acid		
Corn	18.9 ± 0.8 a (16.2%)	27.7 ± 1.6 a (23.7%)	46.6 ± 1.7 a (39.9%)	70.2 ± 2.4 a (60.1%)	117 ± 4.1 a
Wheat	nd	6.0 ± 0.7 b (33.1%)	6.0 ± 0.7 b (33.1%)	12.1 ± 0.3 d (66.9%)	18.1 ± 0.9 d
Barley	nd	6.7 ± 0.06 b (261%)	6.7 ± 0.06 b (261%)	19.0 ± 1.9 c (73.9%)	25.7 ± 1.9 c
Rice	nd	7.4 ± 0.2 b (18.5%)	7.4 ± 0.2 b (18.5%)	32.7 ± 0.8 b (81.5%)	40.1 ± 0.9 b
Oats	7.1 ± 0.2 b (17.5%)	7.0 ± 0.4 b (17.3%)	14.1 ± 0.4 b (34.8%)	26.4 ± 3.0 b (65.2%)	40.5 ± 2.7 b
Quinoa	nd	5.7 ± 0.03 b (30.7%)	5.7 ± 0.03 b (30.7%)	13.1 ± 1.0 d (70.0%)	18.7 ± 0.9 d
Buckwheat	nd	nd	nd	6.3 ± 0.4 e (100%)	6.3 ± 0.4 f
Amaranth	nd	6.3 ± 0.2 b (47.9%)	6.3 ± 0.2 b (47.9%)	6.8 ± 0.2 e (51.9%)	13.1 ± 0.4 e

[a]Sum of free and soluble-conjugated fractions

[b]Sum of free, soluble-conjugated, and insoluble-bound fractions

Table A.3 – Hydroxybenzoic acid-derivative content of grains. Values reported as μmol/100 g (mean ± SD, n = 3). Values with no letter in common within each column are significantly different (p < 0.05). Percent contribution to the whole seed is in parentheses. nd – not detected

	Free	Soluble-Conjugated	Total Soluble[a]	Insoluble-Bound	Total[b]
			p-Hydroxybenzoic Acid		
Corn	14.1 ± 1.3 (35.8%)	14.2 ± 0.8 a (36.0%)	28.2 ± 2.1 a (71.6%)	11.2 ± 0.9 b (28.4%)	39.4 ± 1.3 a
Wheat	15.3 ± 1.2 (50.0%)	nd	15.3 ± 1.2 (50.0%)	15.2 ± 1.2 a (50.0%)	30.6 ± 2.2 b
Barley	nd	11.5 ± 0.6 b (100%)	11.5 ± 0.6 b (100%)	nd	11.5 ± 0.6 c
Rice	nd	nd	nd	nd	nd
Oats	nd	nd	nd	nd	nd
Quinoa	nd	nd	nd	nd	nd
Buckwheat	nd	nd	nd	nd	nd
Amaranth	nd	nd	nd	nd	nd
			Vanillic Acid		
Corn	nd	13.9 ± 0.5 ab (100%)	13.9 ± 0.5 ab (100%)	nd	13.9 ± 0.5 c
Wheat	22.0 ± 2.0 (41.9%)	11.3 ± 0.4 b (21.5%)	33.2 ± 2.4 a (65.0%)	19.2 ± 11.2 (36.6%)	52.5 ± 9.8 a
Barley	nd	nd	nd	nd	nd
Rice	nd	15.5 ± 3.0 a (100%)	15.5 ± 3.0 a (100%)	nd	15.5 ± 3.0 b
Oats	nd	nd	nd	nd	nd
Quinoa	nd	nd	nd	nd	nd
Buckwheat	nd	nd	nd	nd	nd
Amaranth	nd	nd	nd	nd	nd

[a]Sum of free and soluble-conjugated fractions

[b]Sum of free, soluble-conjugated, and insoluble-bound fractions

Table A.4 – Flavonoid content of grains. Values reported as μmol/100 g (mean ± SD, n = 3). Values with no letter in common within each column are significantly different (p < 0.05). Percent contribution to the whole seed is in parentheses. nd – not detected

	Free	Soluble-Conjugated	Total Soluble[a]	Insoluble-Bound	Total[b]
			Rutin		
Quinoa	71.5 ± 4.0 a (95.0%)	3.8 ± 0.6 (5.0%)	75.3 ± 3.4 a (100%)	nd	75.3 ± 3.4 a
Buckwheat	18.2 ± 1.4 b (100%)	nd	18.2 ± 1.4 b (100%)	nd	18.2 ± 1.4 b
Amaranth	nd	nd	nd	nd	nd
			Quercetin		
Quinoa	3.7 ± 0.3 (100%)	nd	3.7 ± 0.3 (100%)	nd	3.7 ± 0.3
Buckwheat	nd	nd	nd	nd	nd
Amaranth	nd	nd	nd	nd	nd

[a]Sum of free and soluble-conjugated fractions

[b]Sum of free, soluble-conjugated, and insoluble-bound fractions

A.3.3 Oxygen Radical Absorbance Capacity

The antioxidant activity was determined using the ORAC assay. ORAC values expressed as μmol Trolox equivalents/100 g grain. Free ORAC ranged from 1530 (amaranth) to 13331 (buckwheat) μmol TE/100 g grain. Bound ORAC ranged from 748 (amaranth) to 10089 (corn) μmol TE/100 g grain. The total ORAC ranged from 2278 (amaranth) to 14273 (corn) μmol TE/100 g grain (**Table A.5**). The total phenolic content of grains was correlated with ORAC ($R^2 = 0.880$. $p < 0.001$).

A.3.4 Cellular Antioxidant Activity of Select Pure Compounds

The cellular antioxidant activity of four phenolic acids found in whole grains was determined using the CAA assay described by Wolfe and Liu (*13*). The concentrations used to determine the CAA did not reduce the number of HepG2 cells by more than 10% compared to the medium control after 24 hours as determined using the modified methylene blue stain assay (*20*). None of the phenolic acids tested inhibited the increase in fluorescence due to DCFH oxidation (**Figure A.3**). Quercetin inhibited the increase in fluorescence due to DCFH oxidation at much lower concentrations than the phenolic acids. The inhibition in fluorescence due to DCFH oxidation was dose dependent for quercetin but not for the four phenolic acids tested.

A.4 Discussion

A.4.1 Total Phenolic Content and Phenolic Composition

The phenolic content of whole grains has been reported previously (*9, 21-24*). The phenolic content values reported in the present study are similar to the values reported in the cited studies. Gorenstein *et al* reported that the total phenolic content of buckwheat, quinoa, and amaranth were 91.2, 60.0, and 43.0 mg gallic acid

Table A.5 – Oxygen radical absorbance capacity of grains. Values expressed as µmol Trolox equivalents/100 g grain (mean ± SD, n = 3).

	Free ORAC	Bound ORAC	Total ORAC[a]
Corn	4185 ± 285	10089 ± 803	14273 ± 992
Barley	4841 ± 171	7081 ± 427	11922 ± 446
Rice	3839 ± 233	2516 ± 122	6536 ± 268
Oats	3237 ± 250	2891 ± 227	6128 ± 312
Wheat	1892 ± 115	2730 ± 554	4623 ± 487
Buckwheat	13331 ± 2986	921 ± 54	14252 ± 2946
Quinoa	4087 ± 545	1641 ± 217	5729 ± 751
Amaranth	1530 ± 107	748 ± 36	2278 ± 75

[a]Sum of free and bound ORAC
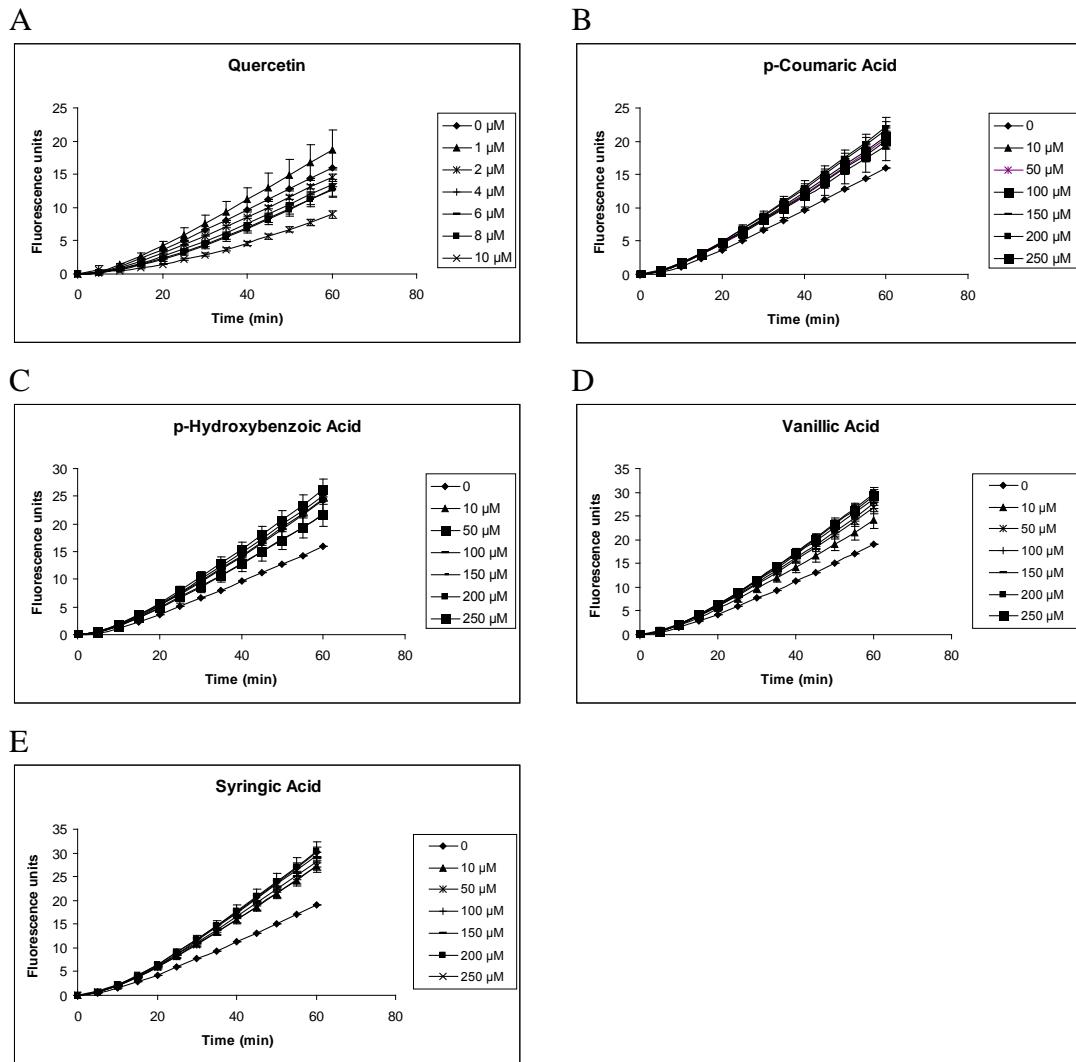
Figure A.3 – Peroxyl radical-induced oxidation of DCFH to DCF in HepG2 cells and the inhibition of oxidation by (A) quercetin, (B) p-coumaric acid, (C) p-hydroxybenzoic acid, (D) vanillic acid, and (E) syringic acid over time. The curves shown in each graph are from a single experiment (mean ± standard deviation, n = 3).

equivalents/100 g (*21*). The total phenolic content of buckwheat, quinoa, and amaranth reported in the present study follows the same general trend.

The phenolic acid composition of grains has been reported previously in several studies (*9, 22, 24-27*). The phenolic acid profile reported in the present study is similar to previously reported studies (*16*). Studies reporting the flavonoid content and composition of whole grains are lacking. The total flavonoid content of wheat, buckwheat, quinoa, and amaranth has been reported previously (*21, 28*). However, the flavonoid composition of each wheat variety was not determined (*28*). It is more important to determine the flavonoid composition of grains because structural differences, degrees of glycosylation, and position of the C-ring can have a significant effect on the cellular antioxidant activity (*15*). Asenstorfer *et al* reported the presence of apigenin-6C-arabinoside-8C-glucoside, apigenin-6C-glucoside-8C-arabinoside, apigenin-6C-arabinoside-8C-galactoside, and apigenin-6C galactoside-8C-arabinoside in the germ fraction of wheat (*29*). The flavonoids rutin and quercetin were detected in the free and insoluble-bound fraction of buckwheat (*27*).

A.4.2 Oxygen Radical Absorbance Capacity

Many studies have determined the antioxidant activity of grains using a variety of different antioxidant activity assays (*6, 9, 11, 21, 27, 30-32*). However, the results from these different antioxidant activity assays are difficult to compare because these methods have not been standardized (*12*). The various phenolic acids found in grains have different oxygen radical absorbance capacities. Yeh *et al* reported that gallic acid had the highest oxygen radical absorbance capacity of the phenolic acids analyzed (*33*). *p*-Coumaric acid had the next highest oxygen radical absorbance capacity, followed by, ferulic acid, and vanillic and syringic acids (*33*). Flavonoids also have a range of oxygen radical absorbance capacities. Of the flavonoids assayed

in the present study, rutin had the highest oxygen radical absorbance capacity followed by apigenin, quercetin, and kaempferol (*15*). The oxygen radical absorbance capacity of flavonoids was not correlated with cellular antioxidant activity (R^2 = 0.214, p > 0.05) (*15*).

A.4.3 Structure Activity Relationships of Flavonoids and Phenolic Acids

Cellular antioxidant activity depends on the structure and glycosylation of the flavonoid, (*15*). Quercetin and kaempferol have the highest cellular antioxidant activity (*15*). The high cellular antioxidant activity of these compounds is due to presence of a hydroxyl group at the 3 position of the C-ring and a keto group at the 4 position of the C-ring. Because quercetin has the highest cellular antioxidant activity, quercetin is used as a standard in the cellular antioxidant activity assay. Quercetin was only detected in the free and/or soluble-conjugated fractions of quinoa and buckwheat (**Table A.4**). No flavonoids were found in the insoluble-bound fraction of any of the grains analyzed (**Table A.4**).

Rutin and apigenin-glycosides have been reported in pseudo-cereals and wheat, respectively (*21, 29*). Wolfe and Liu reported that rutin and apigenin have no cellular antioxidant activity (*15*). Rutin does not have any cellular antioxidant activity because the glycosylation of the 3 position of the C-ring (**Figure A.1**). Apigenin does not have a hydroxyl group at the 3 position of the C-ring (**Figure A.1**). However, apigenin-glycoside and not apigenin were reported in wheat (*29*). The apigenin-glycosides reported in whole wheat have glucose, galactose, and/or arabinose groups attached to the 6 and/or 8 position of the A-ring (*29*). However, these apigenin-glycosides still lack the necessary hydroxyl group at the 3 position of the C-ring.

Phenolic acids have relatively low cellular antioxidant activity compared to flavonoids (**Figure A.4**). Of the phenolic acids found in the insoluble-bound fraction
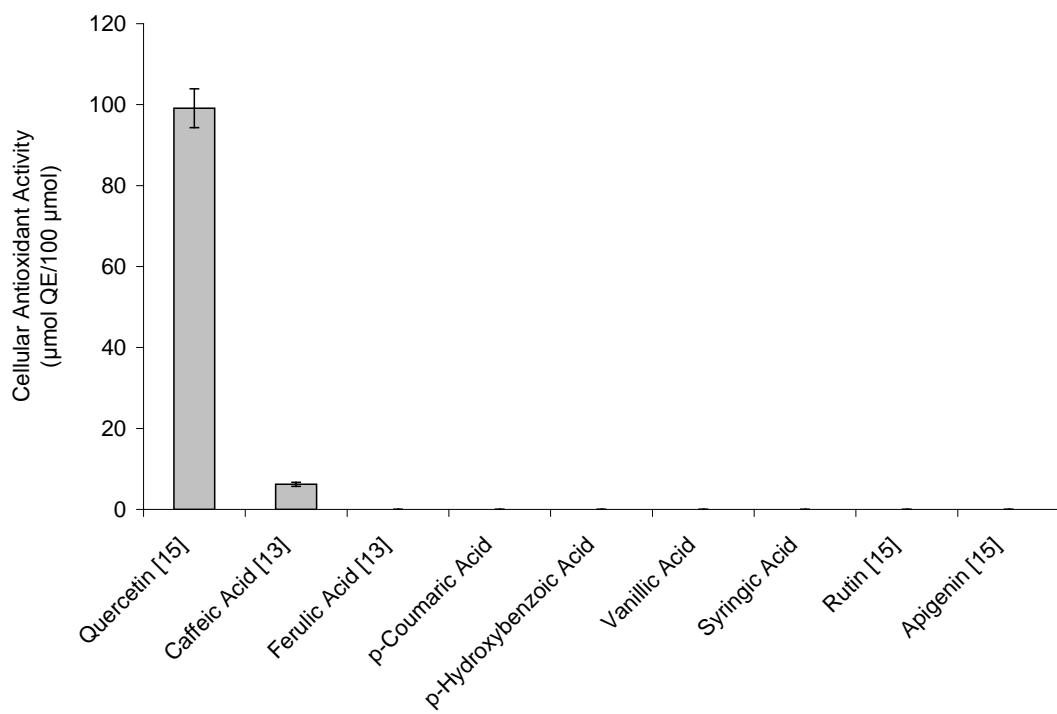
Figure A.4 – Cellular antioxidant activity of whole grain phenolic compounds in HepG2 cells (mean ± standard deviation, n = 3). References are in brackets.

of whole grains, caffeic acid had the highest cellular antioxidant activity. All other phenolic acids had no cellular antioxidant activity (**Figure A.4**). Caffeic acid is the only phenolic acid that has two hydroxyl groups located next to each other on the aromatic ring. This may be due to the structure of caffeic acid. Hydroxylation of the B-ring (**Figure A.1**) of flavonoids was an important factor affecting the cellular antioxidant activity of flavonoids (*15*). Other phenolic acids have either one hydroxyl group (*p*-coumaric acid and *p*-hydroxybenzoic acid) or a combination of hydroxyl and methoxy groups (ferulic acid, syringic acid, and vanillic aicd). Quercetin, the flavonoid with the highest cellular antioxidant activity, has two hydroxyl groups located next to each other on the B-ring (*15*). The structural motif found in caffeic acid is also similar to that found in the C-ring of quercetin, kaempferol, and galangin, another flavonoid with high cellular antioxidant activity (*15*).

A.4.4 Challenges in Determining the Cellular Antioxidant Activity of Whole Grains

Grains contain phenolic compounds that have relatively little or no cellular antioxidant activity (**Figure A.4**). Therefore, it is highly unlikely that phenolic extracts from whole grains will have any cellular antioxidant activity, particularly in the insoluble-bound fraction, which consists entirely of phenolic acids. Phenolics extracts from the insoluble-bound fraction of wheat reduce the cell number by greater than 10% after 24 hours at a concentration of 10 mg/mL in HepG2 cells (*2*). Concentrations of greater than 60 mg/mL of blueberry phenolics extract elicited the same response in HepG2 cells (*13*). The reduction in cell number with the low cellular antioxidant activity of the phenolic compounds in the extract should be indicative of the role of whole grain phenolic compounds in colon cancer prevention.

As I reported in Chapter 3 of this dissertation, relatively low concentrations (10 mg/mL) of phenolics extracts from the insoluble-bound fraction of whole wheat and its milled fractions are able to reduce HepG2 cell numbers by greater than 10% after 24 hours (*2*). This may be because non-epithelial cells are more sensitive to dietary phenolic compounds than cells derived from the intestinal epithelium (*34*). I propose that the use of another cell line, such as the Caco-2 cell line, may be more suitable and more relevant for assessing the cellular antioxidant activity of phenolics extracts from the insoluble-bound fraction of whole grains because Caco-2 cells are derived from the intestinal epithelium making them less sensitive to these phenolic compounds. Further, the role of phenolic compounds from the insoluble-bound fraction of whole grains has been hypothesized to protect against colon cancer by scavenging free radicals (*9*). The use of the Caco-2 cell line would allow for the direct assessment of free radical scavenging capacity of phenolics extracts from the insoluble-bound fraction of whole grains in colon cancer cells. However, the use of the Caco-2 cell line to determine cellular antioxidant activity would not allow for the comparison to the cellular antioxidant activity of previously reported pure compounds or phenolic extracts from fruits as, as the cellular antioxidant activity of pure compounds and phenolics extracts from fruits was determined in HepG2 cells (*13, 14*). Further, the use of another cell line introduces the same lack of standardization observed in other chemical antioxidant activity assays (*12*). I suggest that HepG2 cells should continue to be used in determining the cellular antioxidant activity of phenolics extracts from whole grains.

A.4.5 Summary

The insoluble-bound fraction of whole grains consists mainly of the hydroxycinnamic acids ferulic acid and *p*-coumaric acid and flavonoids (**Table A.2**

and **Table A.4**). It was previously hypothesized that the insoluble-bound fraction of whole grains can prevent against colon cancer by scavenging free radicals in the lower gastrointestinal tract, preventing the initiation of normal cells (*9, 35*). Of the phenolic acids reported in whole grains, only caffeic acid had any cellular antioxidant activity. The cellular antioxidant activity of caffeic acid was relatively low compared to flavonoids such as quercetin and kaempferol. These findings suggest that the potential health benefits of whole grain consumption with regards to reduced risk of colon cancer is independent of the ability of phenolic compounds from the insoluble-bound fraction of whole grains to scavenge free radicals and prevent the initiation of normal cells.

LITERATURE CITED

1.   AACC *AACC Members Agree on Definition of Whole Grain*; AACC St. Paul, MN 1999.

2.   Okarter, N. Whole Wheat Phytochemicals and Potential Health Benefit. Cornell University Ithaca, NY, 2010.

3.   Okarter, N.; Liu, R. H., Health Benefits of Whole Grain Phytochemicals. . *Critical Reviews in Food Science and Nutrition* **2009,** (in press)

4.   Brady, K.; Ho, C. T.; Rosen, R. T.; Sang, S. M.; Karwe, M. V., Effects of processing on the nutraceutical profile of quinoa. *Food Chemistry* **2007,** 100, (3), 1209-1216.

5.   Liu, R. H., Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J Nutr* **2004,** 134, (12 Suppl), 3479S-3485S.

6.   Adom, K. K.; Liu, R. H., Rapid peroxyl radical scavenging capacity (PSC) assay for assessing both hydrophilic and lipophilic antioxidants. *J Agric Food Chem* **2005,** 53, (17), 6572-80.

7.   Cao, G. H.; Alessio, H. M.; Cutler, R. G., Oxygen-Radical Absorbency Capacity Assay for Antioxidants *Free Radical Biology and Medicine* **1993,** 14, (3), 303-311.

8.   Winston, G. W.; Regoli, F.; Dugas, A. J.; Fong, J. H.; Blanchard, K. A., A Rapid Gas Chromatographic Assay for Determining Oxyradical Scavenging Capacity of Antioxidants and Biological Fluids. *Free Radical Biology and Medicine* **1998,** 24, (3), 480-493.

9.   Adom, K. K.; Liu, R. H., Antioxidant activity of grains. *J Agric Food Chem* **2002,** 50, (21), 6182-7.

10.   Zhou, K.; Laux, J. J.; Yu, L., Comparison of Swiss red wheat grain and fractions for their antioxidant properties. *J Agric Food Chem* **2004,** 52, (5), 1118-23.

11.   Moore, J.; Hao, Z.; Zhou, K.; Luther, M.; Costa, J.; Yu, L. L., Carotenoid, tocopherol, phenolic acid, and antioxidant properties of Maryland-grown soft wheat. *J Agric Food Chem* **2005,** 53, (17), 6649-57.

12.   Fardet, A.; Rock, E.; Remesy, C., Is the in vitro antioxidant potential of whole-grain cereals and cereal products well reflected in vivo? *Journal of Cereal Science: 48 (2) 258-276* **2008,** 48, (2), 258-276.

13.   Wolfe, K. L.; Liu, R. H., Cellular Antioxidant Activity (CAA) Assay for Assessing Antioxidants, Foods, and Dietary Supplements. *J Agric Food Chem* **2007**.

14.   Wolfe, K. L.; Kang, X.; He, X.; Dong, M.; Zhang, Q.; Liu, R. H., Cellular Antioxidant Activity of Common Fruits. *J. Agric. Food Chem.* **2008,** 56, (18), 8418-8426.

15.   Wolfe, K. L.; Liu, R. H., Structure/Activity Relationships of Flavonoids in the Cellular Antioxidant Activity Assay. *J. Agric. Food Chem.* **2008,** 56, (18), 8404-8411.

16.   Okarter, N.; Liu, C. S.; Sorrells, M. E.; Liu, R. H., Phytochemical content and antioxidant activity of six diverse varieties of whole wheat. . *Journal of Food Chemistry* **2009,** (in press)

17.   Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M., Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In *Oxidants and Antioxidants, Pt A*, 1999; Vol. 299, pp 152-178.

18.   Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Prior, R. L., High-Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) Using a Multichannel Liquid Handling System Coupled with a Microplate Fluorescence Reader in 96-Well Format. *J. Agric. Food Chem.* **2002,** 50, (16), 4437-4444.

19.   Oliver, M. H.; Harrison, N. K.; Bishop, J. E.; Cole, P. J.; Laurent, G. J., A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. *J Cell Sci* **1989,** 92 (Pt 3), 513-8.

20.   Felice, D. L.; Sun, J.; Liu, R. H., A modified methylene blue assay for accurate cell counting. *Journal of Functional Foods* **2009,** 1, (1), 109-118.

21.   Gorinstein, S.; Vargas, O. J. M.; Jaramillo, N. O.; Salas, I. A.; Ayala, A. L. M.; Arancibia-Avila, P.; Toledo, F.; Katrich, E.; Trakhtenberg, S., The total polyphenols and the antioxidant potentials of some selected cereals and pseudocereals. *European Food Research and Technology* **2007,** 225, (3-4), 321-328.

22.   Li, L.; Shewry, P. R.; Ward, J. L., Phenolic acids in wheat varieties in the HEALTHGRAIN Diversity Screen. *J Agric Food Chem* **2008,** 56, (21), 9732-9.

23.   Liyana-Pathirana, C. M.; Shahidi, F., Antioxidant activity of commercial soft and hard wheat (Triticum aestivum L.) as affected by gastric pH conditions. *J Agric Food Chem* **2005,** 53, (7), 2433-40.

24.   Ward, J. L.; Poutanen, K.; Gebruers, K.; Piironen, V.; Lampi, A. M.; Nystrom, L.; Andersson, A. A. M.; Aman, P.; Boros, D.; Rakszegi, M.; Bedo, Z.; Shewry, P. R., The HEALTHGRAIN Cereal Diversity Screen: Concept, Results, and Prospects. *Journal of Agricultural and Food Chemistry* **2008,** 56, (21), 9699-9709.

25.   Mattila, P.; Pihlava, J. M.; Hellstrom, J., Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products. *J Agric Food Chem* **2005,** 53, (21), 8290-5.

26.   Sosulski, F.; Krygier, K.; Hogge, L., Free, Esterified, and Insoluble-Bound Phenolic-Acids .3. Composition of Phenolic-Acids in Cereal and Potato Flours. *Journal of Agricultural and Food Chemistry* **1982,** 30, (2), 337-340.

27.   Hung, P. V.; Morita, N., Distribution of phenolic compounds in the graded flours milled from whole buckwheat grains and their antioxidant capacities. *Food Chemistry* **2008,** 109, (2), 325-331.

28.   Adom, K. K.; Sorrells, M. E.; Liu, R. H., Phytochemical profiles and antioxidant activity of wheat varieties. *J Agric Food Chem* **2003,** 51, (26), 7825-34.

29.   Asenstorfer, R. E.; Wang, Y.; Mares, D. J., Chemical structure of flavonoid compounds in wheat (Triticum aestivum L.) flour that contribute to the yellow colour of Asian alkaline noodles. *Journal of Cereal Science* **2006,** 43, (1), 108-119.

30.   Liyana-Pathlrana, C. M.; Shahidi, F., Antioxidant and free radical scavenging activities of whole wheat and milling fractions. *Food Chemistry* **2007,** 101, (3), 1151-1157.

31.   Mpofu, A.; Sapirstein, H. D.; Beta, T., Genotype and environmental variation in phenolic content, phenolic acid composition, and antioxidant activity of hard spring wheat. *Journal of Agricultural and Food Chemistry* **2006,** 54, (4), 1265-1270.

32.   Pellegrini, N.; Serafini, M.; Salvatore, S.; Del Rio, D.; Bianchi, M.; Brighenti, F., Total antioxidant capacity of spices, dried fruits, nuts, pulses, cereals and sweets consumed in Italy assessed by three different in vitro assays. *Mol Nutr Food Res* **2006,** 50, (11), 1030-8.

33.   Yeh, C. T.; Yen, G. C., Effects of Phenolic Acids on Human Phenolsulfotransferases in Relation to Their Antioxidant Activity. *J. Agric. Food Chem.* **2003,** 51, (5), 1474-1479.

34.   Janicke, B.; Onning, G.; Oredsson, S. M., Differential effects of ferulic acid and p-coumaric acid on S phase distribution and length of S phase in the human colonic cell line Caco-2. *Journal of Agricultural and Food Chemistry* **2005,** 53, (17), 6658-6665.

35.   Surh, Y.-J., Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **1999,** 428, (1-2), 305-327.

PHENOLICS EXTRACTS FROM THE INSOLUBLE-BOUND FRACTION OF
WHOLE WHEAT BUT NOT REFINED WHEAT INHIBIT THE PROLIFERATION
OF CACO-2 COLON CANCER CELLS

Abstract

The insoluble-bound fraction of two commercial blends of whole and refined wheat (Barretta and Magnolia) was assessed for its total phenolic content, phenolic acid composition, and cytotoxic and antiproliferative effects towards Caco-2 human colon cancer cells. The total phenolic content of Barretta and Magnolia was 97.5 and 95.8 mg gallic acid equivalents/100 g whole wheat, respectively, compared to 13.8 and 12.8 mg gallic acid equivalents/100 g for refined wheat. Ferulic acid was the predominant phenolic acid found in both varieties of whole and refined wheat. *p*-Coumaric acid and caffeic acid were also detected in the insoluble-bound fraction of whole wheat though no caffeic acid was detected in the insoluble-bound fraction of refined wheat. Phenolics extracts from the insoluble-bound fraction of whole wheat were able to significantly ($p < 0.05$) inhibit the proliferation of Caco-2 cells between 24 and 96 hours at a sample concentration of 100 mg/mL. Phenolics extracts from the insoluble-bound fraction of refined wheat did not have similar effects. The sum of the phenolic acids from the insoluble-bound fraction of whole wheat or refined wheat was significantly correlated ($R^2 = 0.9856$) with the antiproliferative activity of the phenolics extracts. The phenolics extracts from the insoluble-bound fraction of whole wheat did not contain phenolic acids at concentrations that elicit antiproliferative activity on their own. This suggests that the phenolic acids found in the insoluble-bound fraction of whole wheat work synergistically with each other to inhibit the proliferation of Caco-2 cells. To my knowledge, this is the first paper to show the

antiproliferative activity of the insoluble-bound fraction of whole wheat in human colon cancer cells. These data also support several epidemiological studies linking increased whole grain consumption and reduced risk of colorectal cancer.

Introduction

A whole grain consists of the intact, ground, cracked or flaked caryopsis, whose principal anatomical components - the starchy endosperm, germ and bran - are present in the same relative proportions as they exist in the intact caryopsis (*1*). The endosperm, the largest component of the whole grain, contains starchy carbohydrates, proteins, vitamins and minerals. Whole grain consumption has been associated with reduced risk of colorectal cancer (*2-4*). The 2005 Dietary Guidelines for Americans recommends Americans to consume at least three ounce-equivalents of whole grain products each day, being sure to "make half your grains whole" (*5*).

Grain processing is a necessary step in the production of grain products and may influence the amounts of phytochemicals found in grains (*6*). Milling is the first processing step in the manufacturing of food products. Milling involves the separation of the germ and bran from the endosperm so that the endosperm can be ground into flour. Prior to milling, the grain is often tempered in order to ease the removal of the germ and bran from the endosperm. The milling of the whole seed generally results in the reduction of macro and micronutrients and is affected by extraction rate (*7*).

Phenolics are compounds with one or more aromatic rings and one or more hydroxyl groups (*8*). The predominant phenolic compounds found in whole grains and whole wheat are phenolic acids (**Figure B.1**). Phenolic acids are hydroxybenzoic-acid and hydroxycinnamic-acid derivatives (*9*). Phenolic acids are generally found esterified or bound to cell wall polymers and are therefore insoluble when extraction
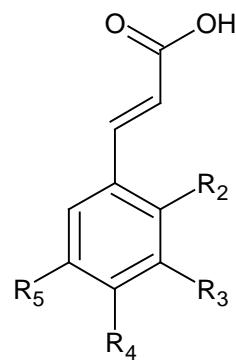
	R_2	R_3	R_4	R_5
Ferulic Acid	H	OMe	OH	H
p-Coumaric Acid	H	H	OH	H
Caffeic Acid	H	OH	OH	H
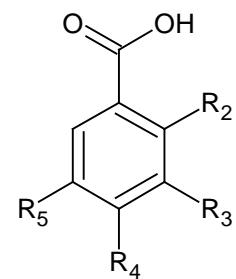
	R_2	R_3	R_4	R_5
p-Hydroxybenzoic Acid	H	H	OH	H
Syringic Acid	H	OMe	OH	OMe
Vanillic Acid	H	OMe	OH	H

Figure B.1. Structure of phenolic acids

solvents are used to extract phenolic compounds from whole or refined grains (*10*). These compounds can be released from the cell wall by alkali or acidic hydrolysis, or enzymatic activity. The total phenolic content and phenolic composition of whole wheat has previously been reported in literature (*11-13*). Andreasen *et al.* proposed a mechanism by which insoluble-bound phenolic compounds may be released in the lower gastrointestinal tract by gut microflora where they may exhibit potential health benefits (*14*). Adom and Liu proposed that this mechanism may partially explain the mechanism of grain consumption in the prevention of colon cancer (*15*). Ferulic acid is the predominant phenolic acid found in whole wheat and is thought to be responsible for the antioxidant activity of whole wheat (*10, 16, 17*) However, the phenolic acids found in the insoluble-bound fraction of whole wheat also have significant ability to inhibit the proliferation of human cancer cell cultures (*18-22*).

Cytotoxicity is the potential of a compound or other treatment to induce cell death (*23*). Cell death can be due to necrosis, apoptosis, or autophagy (*24*). Upon cell death, the integrity of the cell membrane is compromised, causing the cell to leak its contents into the culture medium. Dehydrogenase activity in the culture medium can then be assessed as a marker of cytotoxicity. Lactate dehydrogenase (LDH) activity is commonly used to assess cytotoxicity in a wide range of cell lines *in vitro* (*25-27*).

The reduction in cell number upon treatment with phenolic acids or phenolics extracts may be due to the cytotoxic nature of the treatment and/or the ability of the treatment to inhibit the proliferation of the cancer cells *in vitro*. Various treatments may also inhibit the ability of cancer cells to proliferate *in vitro*. Phenolic compounds may induce cell cycle arrest, leading to a reduced number of cells after a certain period of time when compared to a medium control (*20*). Proliferating cell nuclear antigen (PCNA) is a cofactor of the DNA polymerase system responsible for DNA replication prior to cell division (*28*). PCNA acts as a scaffolding protein by encircling the DNA

161

double helix and recruiting the polymerases. Once attached, the DNA polymerases and PCNA "slide" down the DNA double helix and replicate the DNA. For this reason, PCNA expression is often used as a marker of cell proliferation in studies assessing the proliferation of cancer cells both *in vitro* and *in vivo* (*29-32*).

Though most of the attention has been paid to whole wheat, less attention has been paid to the phenolic acid content and the cytotoxic and antiproliferative effects of refined wheat. Mattila *et al.* (2005) reported the phenolic acid content of refined wheat. Adom *et al.* (2005) reported the total phenolic content and ferulic acid content of refined wheat. Though the two previous studies reported the phenolic content and composition of refined wheat, the total phenolic content and phenolic composition of the insoluble-bound fraction of refined wheat was not reported. Chapter 3 of this thesis reported the presence of phenolic acids in the insoluble-bound fraction of refined wheat (*33*). It is important to determine the total phenolic content, phenolic acid content, and antiproliferative effects of the insoluble-bound fraction of refined wheat, as many of the food products consumed are made from refined wheat. The objectives of this study were 1) to determine the total phenolic content and phenolic acid composition of whole and refined wheat, 2) to determine the cytotoxic and antiproliferative effects of phenolic acids after 24 and 96 hours using Caco-2 colon cancer cells, and 3) to determine the cytotoxic and antiproliferative effects of phenolic extracts from the insoluble-bound fraction of whole and refined wheat in Caco-2 colon cancer cells.

Materials and Methods

Chemicals and Reagents

Sodium hydroxide, hydrochloric acid, and hexanes were purchased from Fisher Scientific (Pittsburgh, PA). Acetone, sodium carbonate, and ethyl acetate were purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Folin-Ciocalteu reagent, ferulic acid, *p*-coumaric acid, caffeic acid, *p*-hydroxybenzoic acid, vannilic acid, and syringic acid were purchased from Sigma (St. Louis, MO). Gallic acid was purchased from ICN Biomedicals, Inc. (Aurora, OH). Methylene blue was purchased from BBL (Cockeysville, MD). Caco-2 cell cultures were purchased from the American Type Culture Collection (ATCC) (Rockville, MD). Delbucco's Modified Eagle's Medium (DMEM), antibiotic/antimycotic, HEPES buffer, and Hanks' Balanced Salt Solution (HBSS) were purchased from Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). All reagents used in the study were analytical or reagent grade.

Samples

Two commercial blends of soft red wheat (Barretta and Magnolia) and their refined flours (refined wheat) were obtained from Lynn Haynes at Kraft Foods (East Hanover, NJ).   The whole seed (whole wheat) was milled to a fine powder using a 20, 40, and 60 mesh size screen successively and mixed thoroughly. All samples were stored at -20˚C.

Extraction of Free Phenolics

One gram of whole or refined wheat flour was blended with 50 mL of 80% chilled acetone. The mixture was then centrifuged at 2500 G for 10 minutes. The

163

supernatant was removed and the remaining pellet was again extracted with 50 mL of 80% chilled acetone. The mixture was again centrifuged at 2500 G for 10 minutes. The supernatants were pooled and evaporated at 45ºC to dryness. The solution was then reconstituted in methanol:hydrochloric acid (1 M, 85:15 v/v), filtered through a 0.45 μm filter, and stored at -40ºC.

Extraction of Insoluble-Bound Phenolics

Insoluble-bound phenolics were extracted from the residue from the free phenolic extraction. The residue was first digested with 2 M sodium hydroxide at room temperature for 1 h whilst shaking under nitrogen. The mixture was then neutralized with concentrated hydrochloric acid. Hexanes were used to extract lipids in the mixture. The remaining mixture was then extracted five times with ethyl acetate. The ethyl acetate fractions were pooled and evaporated to dryness. The phenolics were reconstituted in methanol:hydrochloric acid (1 M, 85:15 v/v), filtered through a 0.45 μm filter, and stored at -40ºC for determination of total phenolic content and phenolic acid content or water for use in cell culture experiments.

Determination of Total Phenolic Content

Total phenolic content of the free and insoluble-bound fraction each whole or refined wheat sample was determined using the colorimetric method described by Singleton *et al.* (*34*). Briefly, phenolics extracts were reacted with Folin-Ciocalteu Reagent and then neutralized with sodium carbonate. After 90 minutes, the absorbance of the resulting solution was measured at 760 nm. Gallic acid was used as the standard and total phenolic content was expressed as mg gallic acid equivalents/100 g sample.

Extraction of Soluble-Conjugated Phenolic Acids

Soluble-conjugated phenolic acids were extracted from free phenolic extracts. Free phenolics extracts were digested with 2 M sodium hydroxide for 1 h at room temperature under nitrogen gas, and then neutralized with concentrated hydrochloric acid. The solution was then extracted five times with ethyl acetate. The ethyl acetate fractions were pooled and evaporated to dryness under nitrogen at 35°C. The remaining residue was dissolved in methanol:hydrochloric acid (1 M, 85:15 v/v), filtered through a 0.45 μm filter, and stored at -40 °C.

Determination of Phenolic Acid Composition

The determination of the phenolic composition was done using an rp-HPLC-DAD method (*35*). Briefly, the mobile phase [water to pH 2.8 with acetic acid (A) and acetonitrile/water 70:30 v/v to pH 2.8 with acetic acid (B)] was delivered using a Waters 600E quaternary pump at a flow rate of 1.5 mL/min using the following gradient program: linear gradient from 0/100% – 10/90% B/A for 2.5 minutes, linear gradient from 10/90% – 12/88% B/A for 3.5 minutes, linear gradient from 12/88% – 23/77% B/A for 10 minutes, linear gradient from 23/77% – 95/5% B/A for 4 minutes, and linear gradient from 95/5% – 0/100% B/A for 6 minutes. The total run time was 20 minutes with a 6 minute delay between injections. Seventy five microliters of sample were injected using a Waters 717 plus autosampler. Separation of phenolic acids was done using a C18 column (5 μm, 250 mm x 4.6 mm; Grace Vydac, Baltimore, MD). Phenolic compounds were detected using a Waters 996 Photodiode Array Detector. Each injection was monitored at 282 nm. Identification of each peak was confirmed using the retention time and absorbance spectrum of each pure compound. Detection limits were determined by analyzing blank measurements according to the method described by Kaiser (*36*). The detection limits of all phenolic

acids were less than 4 µg/mL. Percent recoveries were determined by spiking a known amount of phenolic acid into a sample and performing the extraction and analytical methods. Percent recoveries for all phenolic acids were greater than 80%. Data signals were acquired and processed using Waters Empower software (2002) (Waters Corp., Milford, MA).

Cell Culture

Caco-2 cells were grown in growth medium (DMEM supplemented with 5% FBS, 10 mM HEPES Buffer to pH 7.4, 50 units/mL penicillin, 50 µg/mL streptomycin, and 100 µg/mL gentamicin) and were maintained at 37ºC and 5% CO_2 as described previously (*37, 38*). Cells used in all experiments were less than 45 passages.

Cell Counting Assay

The antiproliferative effects of phenolic acids and insoluble-bound phenolics extracts from whole and refined wheat were assessed in Caco-2 cell cultures using the previously described methylene blue cell counting assay (*39*) modified by our lab (*37*). Caco-2 cells were plated at a density of 4 x 10^4 and 2.5 x 10^4 cells per well to assess cell number after 24 and 96 hours, respectively, and kept at 37˚C in 5% CO_2 for 6 hours. The medium was then replaced by medium containing phenolic acids or insoluble-bound phenolics extracts from whole or refined wheat. The DMSO and HEPES concentrations in the medium containing treatment were 2% and 9.8 mM, respectively. The cells were incubated at 37 ˚C in 5% CO_2 for 24 and 96 hours. After 24 and 96 hours, the medium was removed and cells were rinsed one time with PBS to remove dead and non-adherent cells. The cells were then treated with a fixing/staining solution containing 0.6% methylene blue (BBL, Cockeysville, MD) and 1.25%

166

glutaldehyde (Sigma-Aldrich, St. Louis, MO) in Hank's Balanced Salt Solution (Invitrogen) at 37˚C for 1 hour. Cells were rinsed with water and dried. The methylene blue stain was eluted from the cells with an elution solution containing ethanol/PBS/acetic acid 50:49:1 v/v/v by agitating the plate for 30 minutes. The optical density was measured by a Dynex Technologies MRX-II microplate reader (Dynex Technologies Inc, Chantilly, VA) at 570 nm. The optical density at 570 nm of all controls and treatments was fitted to a standard curve and used to express values as cell number.

Cytotoxicity

The cytotoxic effect of the controls and treatments towards Caco-2 cells was determined using the Non-Radioactive Cytotoxicity Assay Kit provided by Promega (Promega Corporation, Madison, WI). The assay assesses lactate dehydrogenase (LDH) activity in the culture medium as a result of cell lysis induced by the treatment. After treatment with phenolics extracts or controls for 24 or 96 hours, the culture medium was collected. Fifty microliters of substrate/enzyme mix were added to 50 µL of each sample. The solution was incubated at room temperature for 30 minutes protected from light. After 30 minutes, the reaction was stopped and the absorbance of each solution at 490 nm was recorded. Values were reported as percent LDH activity of the medium control.

Cell Treatment for Western Blotting

Caco-2 cells were plated in six-well plates at a density of 5×10^5 cells/well and kept at 37 ˚C in 5% CO_2 for 24 hours. Two wells were used per treatment. The cells were then rinsed with PBS and the culture medium, culture medium containing the phenolics extracts from the insoluble-bound fraction of whole or refined wheat,

culture medium containing the solvent (10% sterile water), or culture medium without FBS was added to the cells.

Preparation of Cell Lysates

Cell lysates were prepared using a modification of a previously reported method (*40*). After 24 or 96 hours, cells were rinsed twice with ice-cold PBS and scraped off the plate using a cell scraper. The cells were then centrifuged at 128 G for 5 minutes at 4˚C. After centrifugation, the cells were suspended in RIPA lysis buffer containing protease inhibitors (1 μg/mL aprotinin; 1 μg/mL leupeptin; 1 μg/mL pepstatin; 400 μM PMSF; 200 μM sodium fluoride; 200 μM sodium orthovanadate). The cell suspension was vortexed for 15 seconds and kept on ice for 20 minutes. The solution was then centrifuged at 15000 G for 15 minutes at 4 ˚C. The supernatants were stored in clean Eppendorf tubes at -80˚C until analysis.

Western Blotting

The total protein content of each sample was determined using a Sigma Total Protein Kit (Sigma-Aldrich, St. Louis, MO). After determination of total protein content, 100 μg total protein from each sample was electrophoresed on a 10% w/v SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Sigma Aldrich, St. Louis, MO) and blocked with 3% w/v nonfat dry milk in PBS for 1 hour at room temperature. The membrane was then incubated overnight with primary antibodies (anti-PCNA, Calbiochem #NA03 (1:2000) and anti-β-actin, Sigma A5316-.2ML (1:2000)) for 24 hours at 4˚C. The corresponding secondary antibody (anti-mouse, Cell Signaling #7076 (1:300)) was applied in 3% w/v nonfat dry milk at room temperature for 1 hour. Blots were revealed using the Phototope HRP Western Blot Detection System (Cell Signaling,

Beverly, MA) and developed using a Kodak Biomax MR Film (Kodak, Rochester, NY). Bands of target proteins were quantified using NIH Image J Software (NIH, Atlanta, GA).

Statistical Analysis

Data were reported as mean ± SD. One-way ANOVA, Fisher's comparison test, and regression analysis were performed using Minitab Statistical Software v. 15 (State College, PA).

RESULTS

Total Phenolic Content of Whole and Refined Wheat

The phenolic content from the free and insoluble-bound fractions of whole and refined wheat was determined using the Folin-Ciocalteu Reagent method described by Singleton *et al.* (*34*). The free phenolic content of Magnolia and Barretta whole wheat was 63.5 and 43.3 mg/100 g, respectively (**Figure B.2**). The free phenolic content of Magnolia and Barretta refined wheat was 33.0 and 24.6 mg/100 g, respectively. The insoluble-bound fraction of whole wheat had a greater total phenolic content than the free fraction (**Figure B.2**). The insoluble-bound phenolic content of Magnolia and Barretta whole wheat was 95.8 and 97.5 mg/100 g, respectively. The insoluble-bound phenolic content of refined what had a lower total phenolic content than the free fraction. The insoluble-bound phenolic content of Magnolia and Barretta refined wheat was 12.8 and 13.8 mg/100 g, respectively.
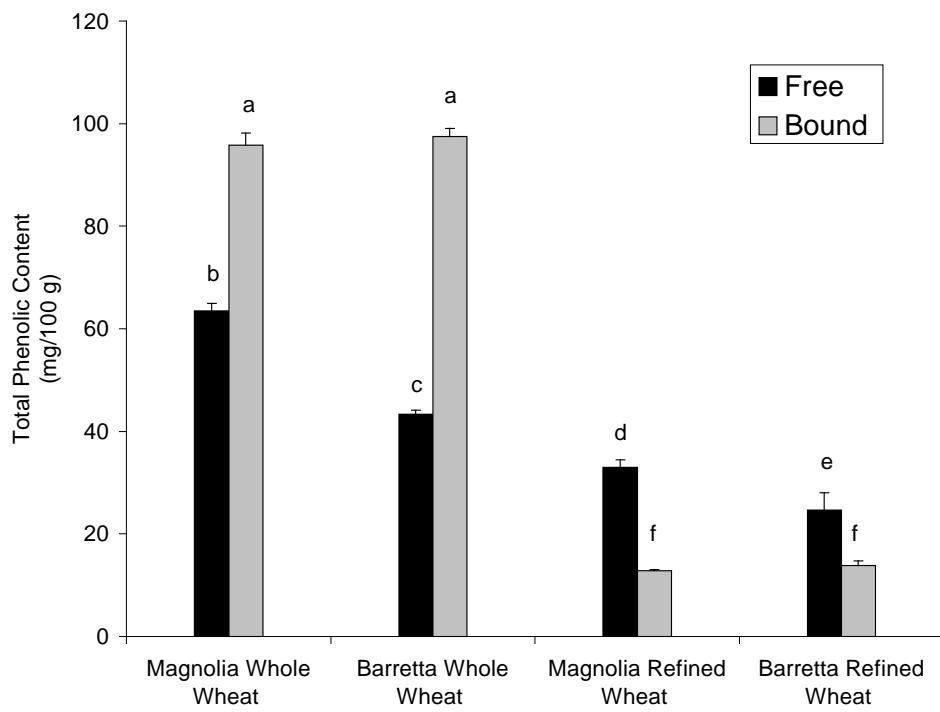
Figure B.2 – Total phenolic content of whole and refined wheat (mean ± standard deviation, n = 3). The total phenolic content of the free and insoluble-bound fraction of whole and refined wheat was determined using a modification of the Folin-Ciocalteu Reagent method (*34, 35*). Bars with no letter in common are significantly different (p < 0.05).

Phenolic Acid Composition of Whole and Refined Wheat

The phenolic acid composition of whole and refined wheat was determined using an rp-HPLC-DAD method. Ferulic acid was found detected in the free, soluble-conjugated, and insoluble-bound fractions of whole and refined wheat (**Table B.1**). *p*-Coumaric acid was detected in the soluble-conjugated and insoluble-bound fractions of whole and refined wheat, with the exception of Magnolia whole wheat, in which *p*-coumaric acid was also found in the free fraction. Syringic acid and vanillic acid were found in the soluble-conjugated fraction of whole and refined wheat. Caffeic acid was only found in the insoluble-bound fraction of whole wheat and was not found in any fraction of refined wheat.

The milling of whole wheat to refined wheat does not result in equal reduction in phenolic composition as can be seen in the representative HPLC chromatograms from the insoluble-bound fraction of whole and refined wheat (**Figure B.3**). The total ferulic acid content of Barretta and Magnolia whole wheat was 335.5 and 325.6 μmol/100 g, respectively. The total ferulic acid content of Barretta and Magnolia refined wheat was 36.6 and 26.8 μmol/100 g, respectively. The insoluble-bound fraction of whole and refined wheat contained greater than 90% and 70% of the total ferulic acid content, respectively. The insoluble-bound ferulic acid content of Barretta and Magnolia whole wheat was 320.2 and 297.0 μmol/100 g, respectively. The insoluble-bound ferulic acid content of Barretta and Magnolia refined wheat was 36.6 and 26.8 μmol/100 g, respectively.

The total *p*-coumaric acid content of Barretta and Magnolia whole wheat was 25.4 and 30.2 μmol/100 g, respectively. These values are significantly ($p < 0.05$) different from one another. The total *p*-coumaric acid content of Barretta and Magnolia refined wheat was 13.5 and 13.1 μmol/100 g, respectively. In both whole and refined wheat, more than half of the total *p*-coumaric acid content was found in
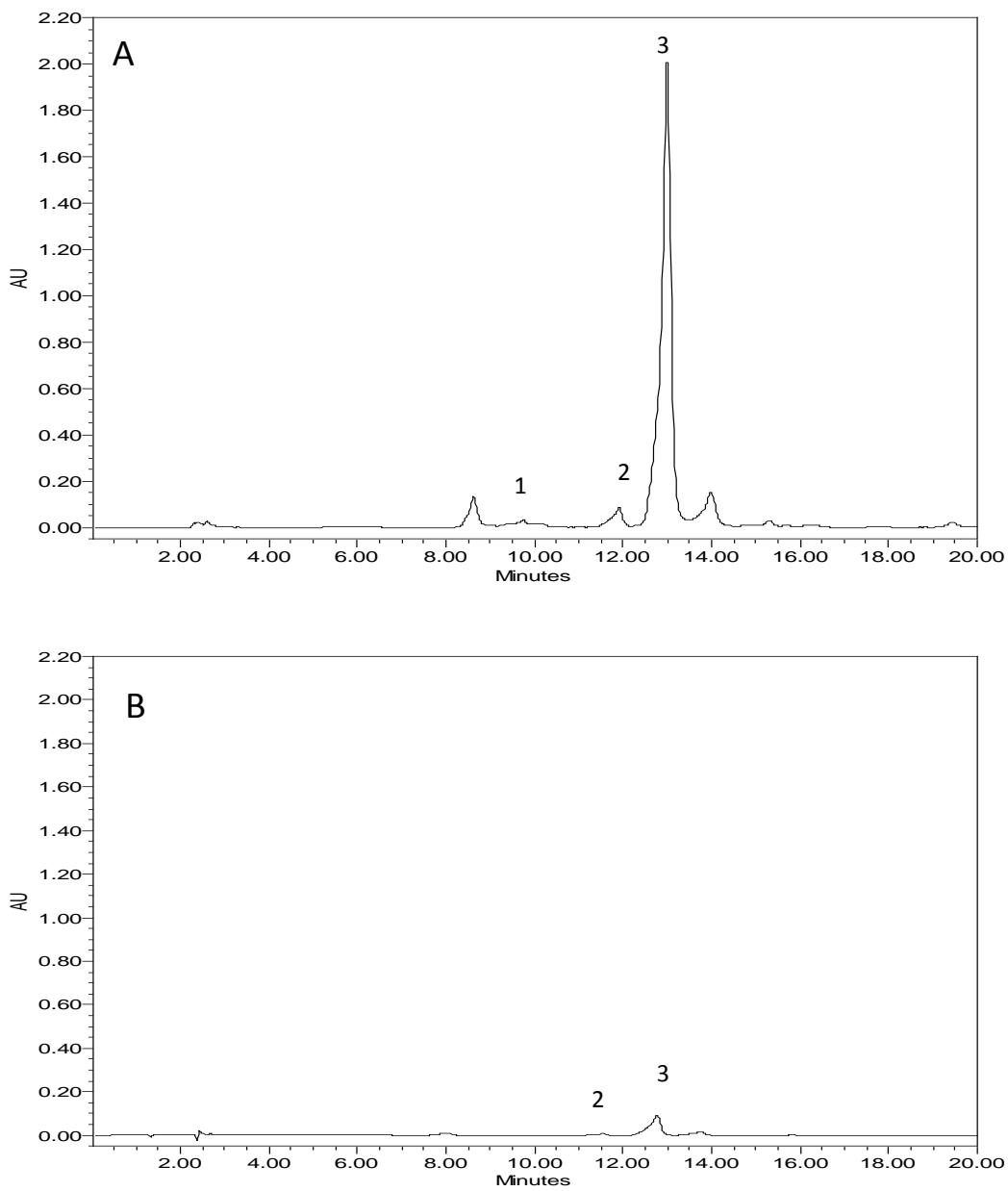
Figure B.3 – Representative HPLC chromatograms from the insoluble-bound fraction of whole wheat (A) and refined wheat (B). Chromatograms were monitored at 282 nm. The following peaks were identified using the retention time and absorbance spectrum of the pure compound: 1, caffeic acid; 2, *p*-coumaric acid; and 3, ferulic acid. See Materials and Methods section for further details on chromatographic separation of phenolic acids.

Table B.1 – Phenolic acid composition of whole and refined wheat. Values expressed as μmol/100 g (mean ± standard deviation, n = 3). Numbers in parentheses represent percent phenolic acid of whole wheat. Values with no letter in common within each column are significantly different (p < 0.05). nd – not detected

	Free	Soluble-Conjugated	Insoluble-Bound	Total
		Ferulic Acid		
Whole Wheat				
Barretta	3.3 ± 1.0 ab	12.0 ± 1.6 b	320 ± 19.4 a	336 ± 20.1 a
Magnolia	3.8 ± 0.6 a	24.8 ± 6.5 a	297 ± 15.5 a	326 ± 17.7 a
Refined Wheat				
Barretta	2.4 ± 0.06 b (72.7%)	3.5 ± 0.2 b (29.1%)	30.8 ± 11.2 b (9.6%)	36.6 ± 11.4 b (10.9%)
Magnolia	2.4 ± 0.05 b (63.2%)	5.2 ± 0.6 b (21.0%)	19.3 ± 5.9 b (6.5%)	26.8 ± 6.0 b (8.2%)
		p-Coumaric Acid		
Whole Wheat				
Barretta	nd	7.1 ± 0.1 a	18.2 ± 1.3 a	25.4 ± 1.2 b
Magnolia	6.2 ± 0.1	7.1 ± 0.2 a	17.0 ± 1.4 a	30.2 ± 1.6 a
Refined Wheat				
Barretta	nd	6.0 ± 0.02 b (84.5%)	7.5 ± 0.03 b (41.2%)	13.5 ± 0.06 c (53.1%)
Magnolia	nd	6.1 ± 0.04 b (94.4%)	7.0 ± 0.3 b (41.2%)	13.1 ± 0.3 c (43.4%)

Table B.1 (continued)

	Free	Soluble-Conjugated	Insoluble-Bound	Total
		Syringic Acid		
Whole Wheat				
Barretta	nd	7.8 ± 1.2 a	nd	7.8 ± 1.2 a
Magnolia	nd	5.4 ± 0.8 b	nd	5.4 ± 0.8 b
Refined Wheat				
Barretta	nd	3.2 ± 0.3 c (41.0%)	nd	3.2 ± 0.3 c (41.0%)
Magnolia	nd	2.6 ± 0.04 c (48.1%)	nd	2.6 ± 0.04 c (48.1%)
		Vanillic Acid		
Whole Wheat				
Barretta	nd	4.7 ± 1.7 a	nd	4.7 ± 1.7 a
Magnolia	nd	3.3 ± 0.5 ab	nd	3.3 ± 0.5 ab
Refined Wheat				
Barretta	nd	1.0 ± 0.1 bc (21.3%)	nd	2.0 ± 0.1 bc (21.3%)
Magnolia	nd	0.7 ± 0.3 c (21.2%)	nd	0.7 ± 0.3 c (21.2%)

174

Table B.1 (continued)

	Free	Soluble-Conjugated	Insoluble-Bound	Total
	Caffeic Acid			
Whole Wheat				
Barretta	nd	nd	8.3 ± 1.0 a	8.3 ± 1.0 a
Magnolia	nd	nd	5.8 ± 1.1 b	5.8 ± 1.1 b
Refined Wheat				
Barretta	nd	nd	nd	nd
Magnolia	nd	nd	nd	nd
	Σ Phenolic Acids			
Whole Wheat				
Barretta	3.3 ± 1.0 b	31.6 ± 3.5 a	347 ± 20.8 a	382 ± 22.5 a
Magnolia	10.0 ± 0.7 a	40.6 ± 7.5 a	320 ± 17.8 a	370 ± 21.3 a
Refined Wheat				
Barretta	2.4 ± 0.06 b (72.7%)	13.6 ± 0.6 b (43.0%)	38.3 ± 11.1 b (11.0%)	54.3 ± 11.5 b (14.2%)
Magnolia	2.4 ± 0.05 b (24.0%)	14.5 ± 1.0 b (35.7%)	26.3 ± 6.2 b (8.2%)	43.1 ± 6.2 b (11.6%)

the insoluble-bound fraction. The insoluble-bound *p*-coumaric acid content of Barretta and Magnolia whole wheat was 18.2 and 17.0 μmol/100 g, respectively. The insoluble-bound *p*-coumaric acid content of Barretta and Magnolia refined wheat was 7.5 and 7.0 μmol/100 g, respectively. The soluble-conjugated *p*-coumaric acid content of Barretta and Magnolia whole wheat was 7.1 μmol/100 g for both wheat varieties. The soluble-conjugated *p*-coumaric acid content of Barretta and Magnolia refined wheat was 6.0 and 6.1 μmol/100 g, respectively.

The syringic acid content of Barretta and Magnolia whole wheat was 7.8 and 5.4 μmol/100 g, respectively. These values were significantly (p < 0.05) different from one another. The syringic acid content of Barretta and Magnolia refined wheat was 3.2 and 2.6 μmol/100 g, respectively. The vanillic acid content of Barretta and Magnolia whole wheat was 4.7 and 3.3 μmol/100 g, respectively. The vanillic acid content of Barretta and Magnolia refined wheat was 1.0 and 0.7 μmol/100 g, respectively.

The caffeic acid content of Barretta and Magnolia whole wheat was 8.3 and 5.8 μmol/100 g, respectively. These values were significantly (p < 0.05) different from one another.

Effect of Phenolic Acids and Phenolics Extracts from Insoluble-Bound Fraction of Whole and Refined Wheat on Caco-2 Cell Number

Caco-2 cells were seeded at different densities and counted using the modified methylene blue stain assay as previously reported (*37*) and described in the Materials and Methods section of this paper. A standard curve was produced and used to quantify cell numbers before and after treatment. The standard curve produced was similar to that reported by Felice *et al.* (*37*) and had an R^2 value of 0.9919 (**Figure B.4**). The growth curve of Caco-2 cells in a 96-well plate showed a significant
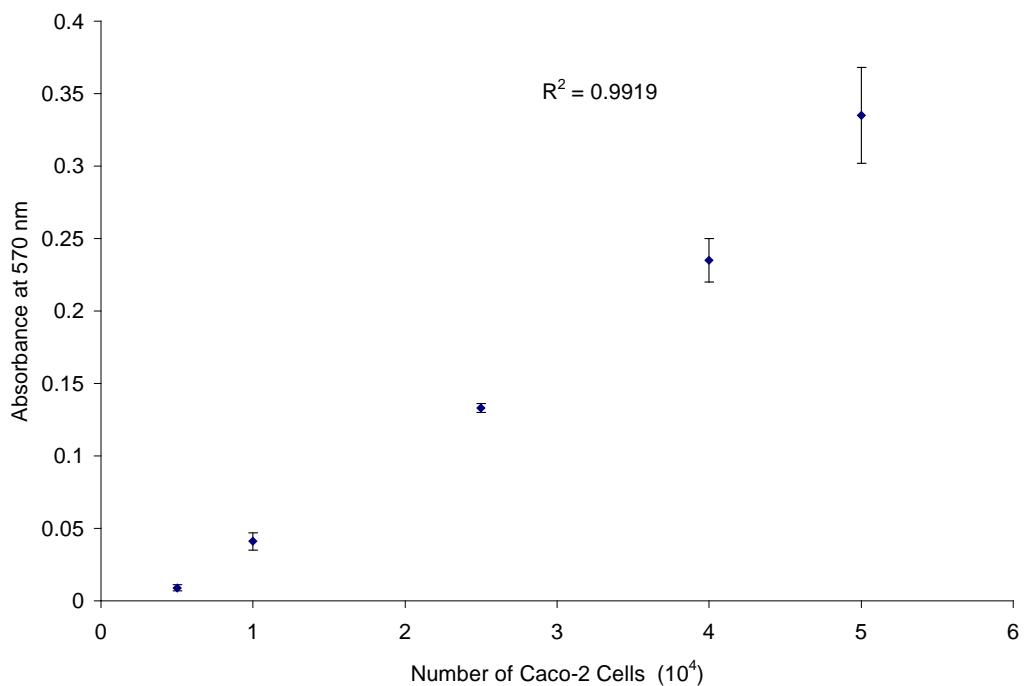
Figure B.4 – Caco-2 cell standard curve. Caco-2 cells were seeded at densities ranging from 0.5 – 5 x 10⁴ cells/well. Six hours later, cells were rinsed with PBS and treated with the methylene blue fixing/staining solution as described in the Materials and Methods section of this paper. The dye was eluted from the cells with elution solution containing ethanol/PBS/acetic acid 50:49:1 v/v/v and the absorbance at 570 nm was determined. The standard curve was used to convert absorbance values of treatments and controls to cell number.

increase in the number of Caco-2 cells after 24 hours when cells are seeded at a density of 4 x 10^4 cells/well (**Figure B.5**). Further, a linear increase in Caco-2 cell number was observed from 0 to 72 hours when cells were seeded at a density of 2.5 x 10^4 cells/well (**Figure B.5**).

Caco-2 cell cultures were treated with ferulic, *p*-coumaric, and caffeic acids at a concentration of 50 µM. The total Caco-2 cell number after treatment with each phenolic acid was assessed using a modification of the methylene blue stain assay after 24 and 96 hours. All phenolic acids (ferulic acid, *p*-coumaric acid, and caffeic acid) significantly ($p < 0.05$) reduced the number Caco-2 cells after 24 compared to the medium control. However, only p-coumaric acid significantly ($p < 0.05$) reduced the number of Caco-2 cells after 96 hours at a concentration of 50 µM. Ferulic acid and caffeic acid significantly ($p < 0.05$) reduced the number of Caco-2 cells after 96 hours at a concentration of 500 µM (**Figure B.6**). The solvent control, 2% DMSO in growth medium, significantly ($p < 0.05$) reduced the number of Caco-2 cells betwee 24 and 96 hours.

Caco-2 cell cultures were treated with phenolics extractions from the insoluble-bound fraction of whole and refined wheat at a concentration of 100 mg/mL. The total Caco-2 cell number after treatment with each phenolic acid was assessed using a modification of the methylene blue stain assay after 24 and 96 hours. Phenolics extracts from the insoluble-bound fraction of whole wheat significantly ($p < 0.05$) reduced the number of Caco-2 cells to 63.0 and 61.6% of the medium control after 24 hours and 77.8 and 78.9% of the medium control after 96 hours for Barretta and Magnolia, respectively (**Table B.2**). Phenolics extracts from the insoluble-bound fraction of refined wheat did not reduce the number of Caco-2 cells between 24 or 96 hours. The solvent control, 10% v/v sterile water in growth medium, did not significantly (p > 0.05) alter cell number between 24 or 96 hours. The FBS control,

(A)
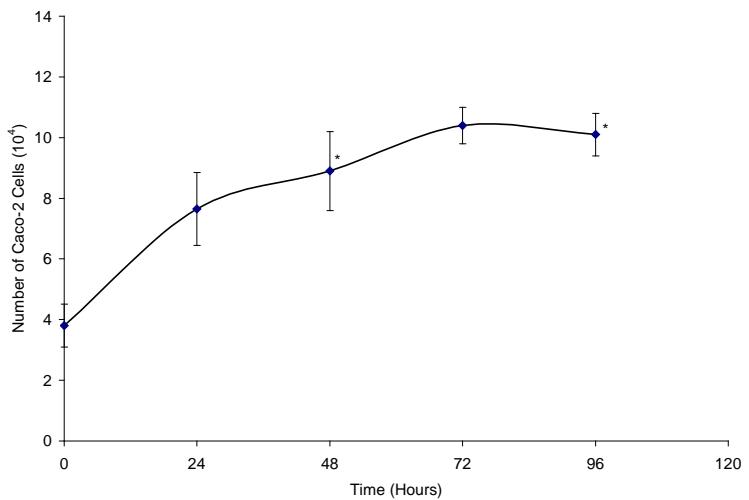
(B)
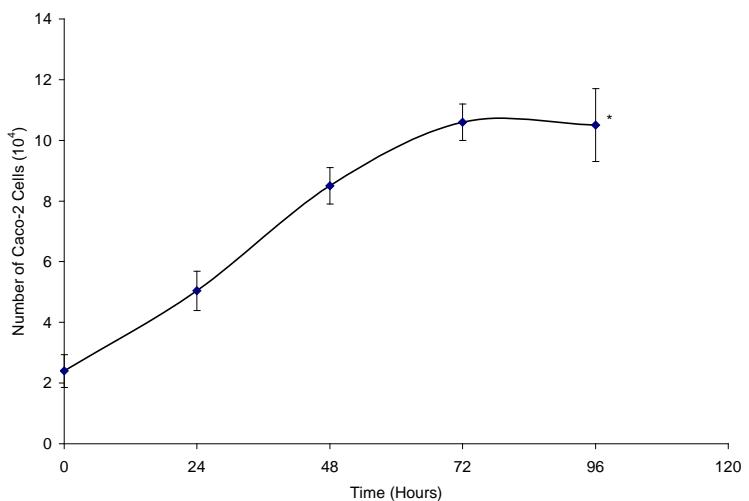
Figure B.5 – Caco-2 cell growth curve in 96-well plate. Caco-2 cells were seeded at densities of 4 x 10^4 (A) and 2.5 x 10^4 (B) cells/well in 5 separate 96-well plates, one plate for each time point. Cells were counted using the modified methylene blue stain cell counting assay described in the Materials and Methods section of this paper. *Indicates no significant difference ($p > 0.05$) from the cell number 24 hours prior.
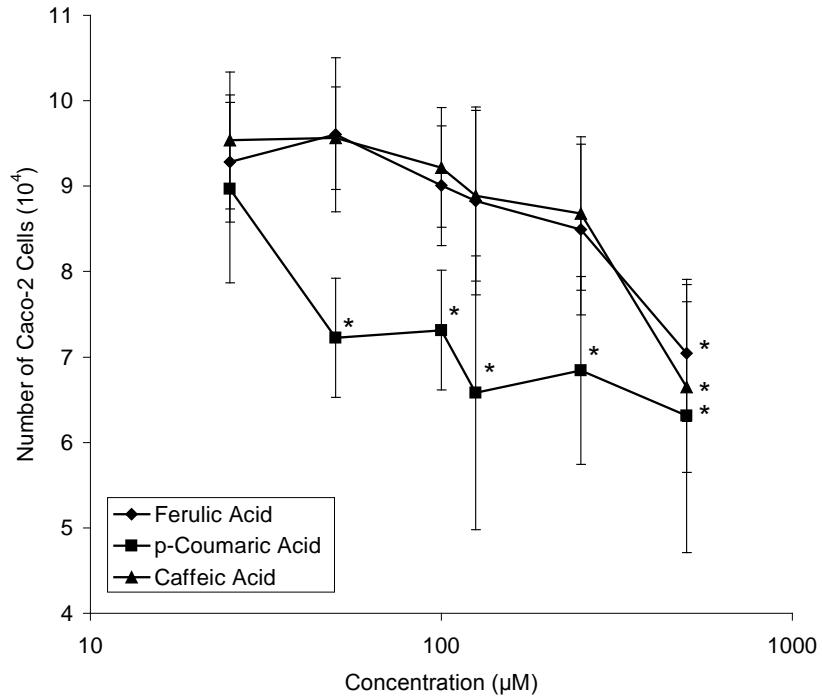
Figure B.6 – Number of Caco-2 cells after 96 hours of treatment with different concentrations of phenolic acids (mean ± standard deviation, n = 3). Caco-2 cells were seeded at a density of 2.5 x 10^4 cells/well. Six hours after seeding, the cells were incubated with phenolic acid in at concentrations ranging from 25 to 500 μM in 2% v/v DMSO in growth medium. The cell number after 96 hours was determined using the modified methylene blue stain assay. *Indicates significant difference from the medium control (p < 0.05).

Table B.2 – Effects of phenolics extracts from the insoluble-bound fraction of whole and refined wheat on proliferation of Caco-2 cell cultures using a modified methylene blue stain assay after 24 and 96 hours. Values reported as cell number (mean ± SD, n ≥ 3). Percent of the medium control is in parentheses.

	24 Hours	96 Hours
	Cell number (10^4)	Cell number (10^4)
Medium Control	7.3 ± 0.7	9.0 ± 0.8
FBS Control §	5.9 ± 0.6 (79.2%)	7.6 ± 0.2 (83.6%)
Solvent Control ‡	7.6 ± 0.6 (104.2%)	9.5 ± 0.9 (104.2%)
Refined Wheat †		
Barretta	7.1 ± 1.3 (96.6%)	8.8 ± 1.1 (97.8%)
Magnolia	7.0 ± 1.6 (96.3%)	8.9 ± 0.9 (99.6%)
Whole Wheat †		
Barretta	4.6 ± 0.2 (59.9%) *	7.0 ± 2.1 (76.6%) *
Magnolia	4.5 ± 1.2 (59.0%) *	7.1 ± 1.4 (78.2%) *

Caco-2 cells were seeded at a density of 4 x 10^4 cells/well for cell counting after 24 hours or 2.5 x 10^4 cells/well for cell counting after 96 hours. Six hours after seeding, the cells were incubated with growth medium, growth medium without FBS, 10% v/v sterile water in growth medium, or 100 mg/mL insoluble-bound phenolics extract in 10% v/v sterile water in growth medium. The cell number after 24 and 96 hours was determined using the modified methylene blue stain assay described in the Materials and Methods section of this paper.

‡ 10% v/v sterile water in growth medium

§ Growth medium without FBS

† Phenolic extracts were delivered at a sample concentration of 100 mg/mL

* Within each column indicates a significant difference from the medium control at $p < 0.05$

growth medium without FBS, reduced the number of Caco-2 cells after 24 and 96 hours but the reduction in cell number was not significant ($p > 0.05$) (**Table B.2**).

LDH Activity

LDH activity was assessed as a marker of cytotoxicity using the Non-Radioactive Cytotoxicity Assay from the Promega Corporation. Cells were treated in the absence of phenol red and at a FBS concentration of 5% v/v or less, as these two factors interfere with optical density readings by providing background absorbance. None of the treatments had significantly more LDH activity than the medium control after 24 hours, though the FBS control and the refined wheat treatment had significantly ($p < 0.05$) less LDH activity than the medium control. After 96 hours, the FBS control, growth medium without FBS, had significantly ($p < 0.05$) more LDH activity than the medium control. The solvent control, 10% sterile water v/v in growth medium, had significantly ($p < 0.05$) less LDH activity (**Figure B.7**). After 24 and 96 hours, the LDH activity of the whole wheat treatment was similar to that of the medium control.

 PCNA Expression

PCNA expression is commonly used as a marker of reduced cell proliferation both *in vitro* and *in vivo* (*29-32*). In this study, PCNA expression was determined using a Western blot method with primary antibodies against PCNA and β-actin. PCNA expression was expressed relative to β-actin expression and the values were reported as a percent of the medium control. There was no significant ($p > 0.05$) change to PCNA expression after 24 or 96 hours in any of the controls or treatments (**Figure B.8**).
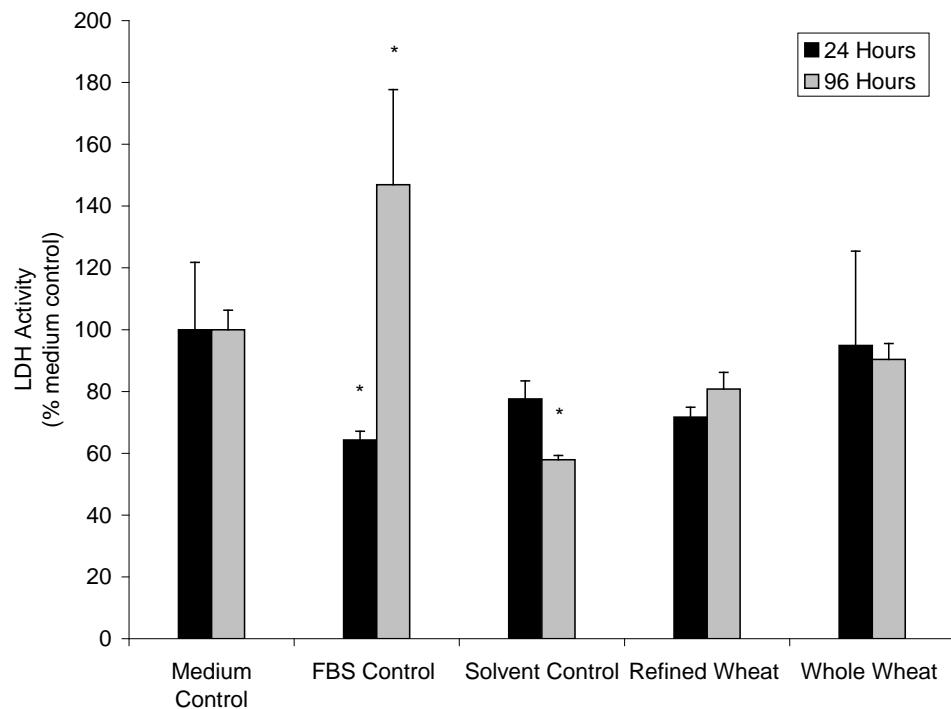
Figure B.7 – Effects of phenolics extracts from the insoluble-bound fraction of whole and refined wheat on LDH Activity in Caco-2 cells. Values reported as percent medium control (mean ± standard deviation, n = 3). Caco-2 cells were seeded at a density of 4 x 10^4 cells/well or 2.5 x 10^4 cells/well in a 96-well plate. Six hours after seeding, the cells were incubated with growth medium, growth medium without FBS, 10% v/v sterile water in growth medium, or 100 mg/mL insoluble-bound phenolics extract in 10% v/v sterile water in growth medium. All controls and treatments were prepared in the absence of phenolic red. After 24 or 96 hours, the medium was collected. LDH activity was determined as described in the Materials and Methods section of this paper. * Indicates a significant difference from the medium control at the respective time point (p < 0.05).
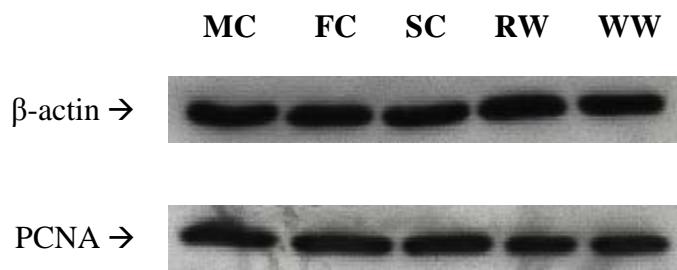
Figure B.8 – Effects of phenolics extracts from the insoluble-bound fraction of whole and refined wheat on PCNA expression after 24 hours. Caco-2 cells were incubated with phenolics extracts from the insoluble-bound fraction of whole or refined wheat (100 mg/mL), growth medium, growth medium without FBS, or 10% v/v sterile water in growth medium. Cytoplasmic fractions were analyzed by Western blotting (*40*) using antibodies against PCNA and β-actin. MC, medium control; FC, FBS control; SC, solvent control; RW, refined wheat (100 mg/mL); WW, whole wheat (100 mg/mL).

DISCUSSION

Total Phenolic Content and Phenolic Acid Composition of Whole and Refined Wheat.

In Chapter 3 of this thesis, I reported the total phenolic content and phenolic acid composition of whole and refined wheat from the free, soluble-conjugated, and insoluble-bound fractions (*33*). In the present study, the total phenolic content and phenolic acid composition of two varieties of whole and refined wheat were reported. The data reported here suggest that the milling of whole wheat to refined wheat reduces the free phenolic content by nearly 45% and the insoluble-bound phenolic content by nearly 85%. The data I report in this study are in general agreement with data I reported in Chapter 3 of this thesis and other studies (*35*).

The effect of milling on individual phenolic acids varies. The milling of whole wheat to refined wheat is responsible for a 92% reduction in the insoluble-bound ferulic acid content, a 59% reduction in the insoluble-bound *p*-coumaric acid content, and the elimination of any caffeic acid found in whole wheat. As the percent reduction of the insoluble-bound phenolic content and the insoluble-bound ferulic acid content are similar, this suggests that ferulic acid is predominant insoluble-bound phenolic compound in whole wheat, a finding which is supported by previous literature (*10, 13*).

Antiproliferative Activities of Phenolic Acids and Grain Extracts.

A modified methylene blue stain assay was used to count cells after treatment with phenolic acids or phenolic extracts from the insoluble-bound fraction of whole wheat and refined wheat. This modified methylene blue stain assay has particular advantages over the more commonly used Trypan Blue stain method because

185

significant ($p < 0.05$) differences in cell number can be observed 24 hours after plating the cells (*37*). Use of the Trypan Blue stain method may indicate a false lag phase in cell growth at 24 hours.

The present study reports the abilities of the phenolic acids found in the insoluble-bound fraction of whole and refined wheat (ferulic acid, p-coumaric acid, and caffeic acid) to reduce Caco-2 cell number after 24 and 96 hours. *p*-Coumaric acid was the most potent inhibitor of cell proliferation after 96 hours and was able to significantly ($p < 0.05$) reduce Caco-2 cells cell numbers after 24 and 96 hours compared to the medium control. Ferulic acid and caffeic acid significantly ($p < 0.05$) reduced the number of Caco-2 cells after 24 hours, however the effect of ferulic acid and caffeic acid on Caco-2 cell number was attenuated after 96 hours. This is consistent with data reported regarding the antiproliferative activity of ferulic acid at 50 µM in HT-29 and SW480 colon cancer cell lines (*19*). Janicke *et al* (2005) reported that *p*-coumaric acid significantly inhibited the proliferation of Caco-2 cells after 24 and 72 hours at 1500 µM. The present study reports the significant ($p < 0.05$) antiproliferative activity of *p*-coumaric acid at 50 µM. Phenolic acids have also been reported to reduce the number of non-epithelial cells but at lower concentration (*18, 21*).

The present study reports the abilities of phenolics extracts from the insoluble-bound fraction of whole and refined wheat to reduce Caco-2 cell number after 24 and 96 hours. Phenolics extracts from the insoluble-bound fraction of whole wheat but not refined wheat reduced the number of Caco-2 cells after 24 and 96 hours compared to the medium control. However, phenolics extracts from the insoluble-bound fraction of refined wheat did not have similar effects on Caco-2 cells after 24 and 96 hours.

Data regarding the antiproliferative effect of phenolics extracts from whole grains or milled fractions of whole grains is lacking. Hudson *et al.* reported that

phenolics extracts from rice bran extract exhibited significant ($p < 0.05$) antiproliferative activity towards two colon cancer cell lines (*19*). In Chapter 3 of this thesis, I reported that phenolics extracts from the insoluble-bound fraction of whole wheat and its milled fractions (bran/shorts, germ, and endosperm) had significant ($p < 0.01$) antiproliferative activity towards HepG2 cells at 10 mg/mL (*33*).

Reduction in Cell Number and Cytotoxic and Antiproliferative Effects towards Caco-2 Cells

In the present study, a cell counting assay, cytotoxicity assay, and Western blotting method were used to assess the effect of phenolics extracts from the insoluble-bound fraction of whole and refined wheat on Caco-2 colon cancer cells. The cell counting assay showed that the number of Caco-2 cells increased 24 and 96 hours after adding fresh culture medium (5% FBS-DMEM) to the cells (**Figure B.5**). When Caco-2 cells were grown in the absence of FBS, the cell number was reduced to 79.2% and 83.6% of the medium control after 24 and 96 hours, respectively (**Table B.2**). The reduction in cell number was accompanied by a significant ($p < 0.05$) increase in LDH activity after 96 hours (**Figure B.7**), indicating a significant increase in cell death. The reduction in cell number and the increase in LDH activity after treatment with culture medium without FBS suggest that the decrease in cell number observed after 96 hours is due to cell death.

Phenolics extracts from the insoluble-bound fraction of whole wheat significantly (p < 0.05) reduced the number Caco-2 cells to 59.5% and 74.7% of the medium control after 24 and 96 hours, respectively (**Table B.2**). However, there was no significant increase in LDH activity in the culture medium after 24 and 96 hours (**Figure B.7**), suggesting that there is no significant increase in cell death. These data suggest that the reduction in cell number upon treatment with phenolics extracts from

the insoluble-bound fraction of whole wheat may not be due to the cytotoxic effects of the treatment.

PCNA expression is generally used as a marker of antiproliferative activity (*29-32*). There was no significant decrease in PCNA expression observed in cells treated with phenolics extracts from the insoluble-bound fraction of whole wheat or culture medium without FBS (**Figure B.8**). It is important to note that the Caco-2 cells still proliferated even after treatment with phenolics extracts from the insoluble-bound fraction of whole wheat or culture medium without FBS (**Table B.2**). This suggests that PCNA is still being expressed in these Caco-2 cells treated with the phenolics extracts or growth medium lacking FBS. Though the expression of PCNA may be decreased in cells treated with phenolics extracts from the insoluble-bound fraction of whole wheat and culture medium without FBS the Western blot method used in the present study may not be able to detect minor changes in PCNA expression. Previous studies have used immunohistochemical and microscopic methods to determine the relative PCNA expression (*29, 41-44*). The combination of immunohistochemistry and microscopy allows for a larger sample size and relative ease of quantification.

The Significance of This Study

The large intestine has a volume of 200 mL (*20*). Therefore, the 100 mg/mL dose of phenolics extract from the insoluble-bound fraction of whole wheat used in the present study is equal to consumption of 20 g whole wheat in the large intestine. Based on the data provided in **Table B.1**, consumption of 20 g whole wheat would provide roughly 308, 18, and 7 μM ferulic acid, *p*-coumaric acid, and caffeic acid to the large intestine, respectively. Further, consumption of 20 g of refined wheat would provide roughly 25 and 7.2 μM ferulic acid and *p*-coumaric acid, respectively. Ferulic

acid, *p*-coumaric acid, and caffeic acid do not have any significant antiproliferative activity in Caco-2 cells at these concentrations. The sum of the phenolic acids in the insoluble-bound fraction of whole or refined wheat (**Table B.1**) is correlated with the Caco-2 cell number expressed as a percent of the medium control after 96 hours ($R^2 =$ 0.9856). These data together suggest that the reduction in cell number after treatment with phenolics extracts from the insoluble-bound fraction of whole wheat is due to the synergistic role of phenolic acids in the inhibition of cell proliferation as previously suggested (*8*).

In Chapter 3 of this thesis, I reported that insoluble-bound fraction of whole wheat and its milled fractions (bran/shorts, germ, and endosperm) had significant (p < 0.01) antiproliferative activity at 10 mg/mL in HepG2 cells (*33*). These data suggest that both whole wheat and refined wheat are able to protect against cancer by inhibiting the proliferation of cancer cells. It is important to note that cells derived from an environment that is high in dietary components may be able to tolerate higher concentrations of dietary compounds (*20*). This may partially explain why relatively low concentrations of phenolic acids and phenolics extracts were able to inhibit the proliferation of T47D breast cancer cells and ECV304 umbilical vein endothelial cells (*18, 21*). Based on these studies and the data I report in this thesis, I suggest that cells derived from the intestinal epithelium should be used to investigate the potential of phenolics extracts from the insoluble-bound fraction of grains to protect against colon cancer.

Summary

The milling of whole grains to refined grains is an important step in the production of grain based food products. As a result of milling, phenolic compounds from the insoluble-bound fraction of the grain are lost. The present study shows that

phenolic extracts from the insoluble-bound fraction of whole wheat, but not refined wheat, can significantly ($p < 0.05$) reduce the number of Caco-2 colon cancer cells after 24 and 96 hours compared to the medium control. This reduction in cell number is not due to the cytotoxic effects of the treatments. The reduction in cell number may be due to the antiproliferative effects of the treatments. To our knowledge, this is the first study to report the antiproliferative activity of the insoluble-bound fraction of whole or refined wheat in a human colon cancer cell line. Though the exact mechanism is unclear, the data suggest that phenolic acids found in the insoluble-bound fraction of whole wheat work synergistically to inhibit the proliferation of colon cancer cells. The data presented in the paper support the hypothesis that whole grain consumption may prevent against colon cancer compared to refined grains and also support numerous epidemiological studies that have linked increased whole grain consumption to reduced risk of colon cancer (*2-4*). The present study underscores the importance of whole grain consumption.

LITERATURE CITED

1.   AACC *AACC Members Agree on Definition of Whole Grain*; AACC St. Paul, MN 1999.

2.   Jacobs, D. R., Jr.; Slavin, J.; Marquart, L., Whole grain intake and cancer: a review of the literature. *Nutr Cancer* **1995,** 24, (3), 221-9.

3.   Larsson, S. C.; Giovannucci, E.; Bergkvist, L.; Wolk, A., Whole grain consumption and risk of colorectal cancer: a population-based cohort of 60 000 women. *British Journal of Cancer* **2005,** 92, (9), 1803-1807.

4.   Schatzkin, A.; Mouw, T.; Park, Y.; Subar, A. F.; Kipnis, V.; Hollenbeck, A.; Leitzmann, M. F.; Thompson, F. E., Dietary fiber and whole-grain consumption in relation to colorectal cancer in the NIH-AARP Diet and Health Study. *Am J Clin Nutr* **2007,** 85, (5), 1353-60.

5.   USDA *Dietary Guidelines for Americans 2005*; USDA Human Nutrition Information Service Hyattsville, MD, 2005.

6.   Slavin, J. L.; Jacobs, D.; Marquart, L., Grain processing and nutrition. *Crit Rev Biotechnol* **2001,** 21, (1), 49-66.

7.   Dewettinck, K.; Van Bockstaele, F.; Kuhne, B.; de Walle, D. V.; Courtens, T. M.; Gellynck, X., Nutritional value of bread: Influence of processing, food interaction and consumer perception. *Journal of Cereal Science* **2008,** 48, (2), 243-257.

8.   Liu, R. H., Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J Nutr* **2004,** 134, (12 Suppl), 3479S-3485S.

9.   Okarter, N.; Liu, R. H., Health Benefits of Whole Grain Phytochemicals. . *Critical Reviews in Food Science and Nutrition* **2009,** (in press)

10.   Sosulski, F.; Krygier, K.; Hogge, L., Free, Esterified, and Insoluble-Bound Phenolic-Acids .3. Composition of Phenolic-Acids in Cereal and Potato Flours. *Journal of Agricultural and Food Chemistry* **1982,** 30, (2), 337-340.

11.   Adom, K. K.; Sorrells, M. E.; Liu, R. H., Phytochemical profiles and antioxidant activity of wheat varieties. *J Agric Food Chem* **2003,** 51, (26), 7825-34.

12.   Mattila, P.; Pihlava, J.-m.; Hellstrom, J., Contents of Phenolic Acids, Alkyl- and Alkenylresorcinols, and Avenanthramides in Commercial Grain Products. *Journal of Agricultural and Food Chemistry* **2005,** 53, (21), 8290-8295.

13.   Moore, J.; Hao, Z.; Zhou, K.; Luther, M.; Costa, J.; Yu, L. L., Carotenoid, tocopherol, phenolic acid, and antioxidant properties of Maryland-grown soft wheat. *J Agric Food Chem* **2005,** 53, (17), 6649-57.

14.   Andreasen, M. F.; Kroon, P. A.; Williamson, G.; Garcia-Conesa, M. T., Intestinal release and uptake of phenolic antioxidant diferulic acids. *Free Radic Biol Med* **2001,** 31, (3), 304-14.

15.   Adom, K. K.; Liu, R. H., Antioxidant activity of grains. *J Agric Food Chem* **2002,** 50, (21), 6182-7.

16.   Mateo Anson, N.; van den Berg, R.; Havenaar, R.; Bast, A.; R. M. M. Haenen, G., Ferulic Acid from Aleurone Determines the Antioxidant Potency of Wheat Grain (Triticum aestivum L.). *Journal of Agricultural and Food Chemistry* **2008,** 56, (14), 5589-5594.

17.   Shahidi, Methods of Analysis and Quantification of Phenolic Compounds. In *Food Phenolics: Sources, chemistry, effects, and applications*, Technomic Publishing Company, Inc.: Lancaster, PA, 1995.

18.   Hou, Y. Z.; Yang, J.; Zhao, G. R.; Yuan, Y. J., Ferulic acid inhibits endothelial cell proliferation through NO down-regulating ERK1/2 pathway. *Journal of Cellular Biochemistry* **2004,** 93, (6), 1203-1209.

19.   Hudson, E. A.; Dinh, P. A.; Kokubun, T.; Simmonds, M. S. J.; Gescher, A., Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. *Cancer Epidemiology Biomarkers & Prevention* **2000,** 9, (11), 1163-1170.

20.   Janicke, B.; Onning, G.; Oredsson, S. M., Differential effects of ferulic acid and p-coumaric acid on S phase distribution and length of S phase in the human colonic cell line Caco-2. *Journal of Agricultural and Food Chemistry* **2005,** 53, (17), 6658-6665.

21.   Kampa, M.; Alexaki, V. I.; Notas, G.; Nifli, A. P.; Nistikaki, A.; Hatzoglou, A.; Bakogeorgou, E.; Kouimtzoglou, E.; Blekas, G.; Boskou, D.; Gravanis, A.; Castanas, E., Antiproliferative and apoptotic effects of selective phenolic acids on T47D human breast cancer cells: potential mechanisms of action. *Breast Cancer Research* **2004,** 6, (2), R63-R74.

22.   Romier, B.; Van De Walle, J.; During, A.; Larondelle, Y.; Schneider, Y. J., Modulation of signalling nuclear factor-kappaB activation pathway by polyphenols in human intestinal Caco-2 cells. *Br J Nutr* **2008,** 100, (3), 542-51.

23.   Eisenbrand, G.; Pool-Zobel, B.; Baker, V.; Balls, M.; Blaauboer, B. J.; Boobis, A.; Carere, A.; Kevekordes, S.; Lhuguenot, J. C.; Pieters, R.; Kleiner, J., Methods of in vitro toxicology. *Food and Chemical Toxicology* **2002,** 40, (2-3), 193-236.

24.   Kroemer, G.; Galluzzi, L.; Vandenabeele, P.; Abrams, J.; Alnemri, E. S.; Baehrecke, E. H.; Blagosklonny, M. V.; El-Deiry, W. S.; Golstein, P.; Green, D. R.; Hengartner, M.; Knight, R. A.; Kumar, S.; Lipton, S. A.; Malorni, W.; Nunez, G.; Peter, M. E.; Tschopp, J.; Yuan, J.; Piacentini, M.; Zhivotovsky, B.; Melino, G., Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death and Differentiation* **2009,** 16, (1), 3-11.

25.   Patil, A. J.; Gramajo, A. L.; Sharma, A.; Seigel, G. M.; Kuppermann, B. D.; Kenney, M. C., Differential effects of nicotine on retinal and vascular cells in vitro. *Toxicology* **2009,** 259, (1-2), 69-76.

26.   Polanco, M. J.; Alguacil, L. F.; Albella, B.; Segovia, J. C.; González-Martín, C., Yohimbine prevents the effect of morphine on the redox status of neuroblastoma × glioma NG108-15 cells. *Toxicology Letters* **2009,** 189, (2), 115-120.

27.   Singer, C. A.; Figueroa-Masot, X. A.; Batchelor, R. H.; Dorsa, D. M., The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. *Journal of Neuroscience* **1999,** 19, (7), 2455-2463.

28.   Moldovan, G. L.; Pfander, B.; Jentsch, S., PCNA, the maestro of the replication fork. *Cell* **2007,** 129, (4), 665-679.

29.   Kaczmarek, E.; Lacka, K.; Majewski, P.; Trzeciak, P.; Gorna, A.; Jarmolowska-Jurczyszyn, D.; Kluk, A., Selected markers of proliferation and apoptosis in the parathyroid lesions: a spatial visualization and quantification. *Journal of Molecular Histology* **2008,** 39, (5), 509-517.

30.   Lee, S.-J.; Lim, K.-T., Phytoglycoprotein (24 kDa) inhibits expression of PCNA via PKC[alpha] and MAPKs in oxygen radical-stimulated Chang liver cells. *The Journal of Nutritional Biochemistry* **2009,** 20, (2), 96-105.

31.   Marotta, F.; Yadav, H.; Pathak, S.; Minelli, E.; Signorelli, P.; Lorenzetti, A.; Marandola, P., Inhibition of Human Breast Cancer Cell Growth and Enzymatic Activity by a Fermented Nutraceutical. *Annals of the New York Academy of Sciences* **2009,** 1155, (Steroid Enzymes and Cancer), 273-277.

32.   Yibo, W.; Yi, Z.; Fang, L.; Xiaohan, F.; Jinxing, C.; Channa, Z.; Rutai, H., KCTD10 interacts with proliferating cell nuclear antigen and its down-regulation could inhibit cell proliferation. *Journal of Cellular Biochemistry* **2009,** 106, (3), 409-413.

33.   Okarter, N. Whole Wheat Phytochemicals and Potential Health Benefit. Cornell University Ithaca, NY, 2010.

34.   Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M., Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. In *Oxidants and Antioxidants, Pt A*, 1999; Vol. 299, pp 152-178.

35.   Okarter, N.; Liu, C. S.; Sorrells, M. E.; Liu, R. H., Phytochemical content and antioxidant activity of six diverse varieties of whole wheat. . *Journal of Food Chemistry* **2009,** (in press)

36.   Kaiser, H., Quantitation in Elemental Analysis. *Analytical Chemistry* **1970,** 4, 24A - 59A

37.   Felice, D. L.; Sun, J.; Liu, R. H., A modified methylene blue assay for accurate cell counting. *Journal of Functional Foods* **2009,** 1, (1), 109-118.

38.   Liu, C. S.; Glahn, R. P.; Liu, R. H., Assessment of Carotenoid Bioavailability of Whole Foods Using a Caco-2 Cell Culture Model Coupled with an in Vitro Digestion. *J. Agric. Food Chem.* **2004,** 52, (13), 4330-4337.

39.   Oliver, M. H.; Harrison, N. K.; Bishop, J. E.; Cole, P. J.; Laurent, G. J., A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. *J Cell Sci* **1989,** 92 (Pt 3), 513-8.

40.   Yoon, H.; Liu, R. H., Effect of selected phytochemicals and apple extracts on NF-kappaB activation in human breast cancer MCF-7 cells. *J Agric Food Chem* **2007,** 55, (8), 3167-73.

41.   Emenaker, N. J.; Calaf, G. M.; Cox, D.; Basson, M. D.; Qureshi, N. In *Short-chain fatty acids inhibit invasive human colon cancer by modulating uPA, TIMP-1, TIMP-2, mutant p53, Bcl-2, Bax, p21 and PCNA protein expression in an in vitro cell culture model*, 2001; Amer Inst Nutrition: 2001; pp 3041S-3046S.

42.   Liu, J. R.; Dong, H.; Chen, B.; Zhao, P.; Liu, R. H., Fresh apples suppress mammary carcinogenesis and proliferative activity and induce apoptosis in mammary tumors of the Sprague-Dawley rat. *Journal of Agricultural and Food Chemistry* **2009,** 57, (1), 297-304.

43.   Yao, M.; Lam, E. C.; Kelly, C. R.; Zhou, W.; Wolfe, M. M., Cyclooxygenase-2 selective inhibition with NS-398 suppresses proliferation and invasiveness and delays liver metastasis in colorectal cancer. *British Journal of Cancer* **2004,** 90, (3), 712-719.

44.   Zopf, S.; Neureiter, D.; Bouralexis, S.; Abt, T.; Glaser, K. B.; Okamoto, K.; Ganslmayer, M.; Hahn, E. G.; Herold, C.; Ocker, M., Differential response of p53 and p21 on HDAC inhibitor-mediated apoptosis in HCT116 colon cancer cells in vitro and in vivo. *International Journal of Oncology* **2007,** 31, (6), 1391-1402.