ABSTRACT

Title CHEMICAL COMPOSITIONS, ANTIOXIDANT AND ANTIPROLIFERATIVE PROPERTIES OF WHEAT

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To promote its use in nutraceutical and functional ingredients, different growing environments, genotypes, and processing fractions of soft wheat (*Triticum aestivum* L.) were investigated for their chemical compositions and biological activities. The first study of this research investigated phytochemicals composition and antiproliferative activities of ten wheat bran samples. It was found that the individual wheat bran samples significantly differed in their chemical and biological properties. The second part of this research further studied influences of genotype (G), growing environment (E), and their interaction (G × E) on the phytochemical compositions and antioxidant properties of the same ten wheat bran cultivars. The results indicated that larger variability for health beneficial components and antioxidant properties of soft winter wheat bran were attributed more by E than G and G ×E. The third study was to investigate phytochemical profiles and antiproliferative properties of bread processing fractions (dough, crumb, and upper crust) from refined and whole-wheat flours. The results showed that baking reduced the concentrations of carotenoids and tocopherols, however, the upper crust fraction had significant higher

levels of phenolic acids than in the dough and crumb fractions, suggesting that total phenolic acids content might not decrease during baking breads made from refined and whole wheat flours.

CHEMICAL COMPOSITIONS, ANTIOXIDANT AND ANTIPROLIFERATIVE PROPERTIES OF WHEAT

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List of Abbreviations

AAPH	2, 2'-azinobis (2-amidinopropane) dihydrochloride
$ABTS^{\bullet+}$	2, 2'azinobi-3-ethylbenzothiazoline-6-sulfonic acid
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BE	Bran equivalent
CAA	Cellular antioxidant activity
CHD	Coronary heart disease
CVD	Cardiovascular disease
DCFHDA	2', 7'-dichlorofluorescein diacetate
DMSO	Dimethyl sulfoxide
$DPPH^{\bullet}$	2, 2-diphenyl-1-picrylhydrazyl
EDTA	Disodium ethylenediaminetetraacetate
FAE	Ferulic acid equivalents
FL	Fluorescein
GAE	Gallic acid equivalents
GLM	General linear model
GPx	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HOSC	Hydroxyl radical scavenging capacity
HPLC	High performance liquid chromatography
LDL	Low-density lipoprotein
$^{\bullet}NO$	Nitric oxide
$^{\bullet}NO_2$	Nitrogen dioxide
RS	Reactive Species
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
$ROO^{\bullet}$	Peroxyl radicals
SOD	Superoxide dismutase
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic contents
$O_2^{\bullet-}$	Anion radical
$^1O_2$	Singlet oxygen
$^{\bullet}OH$	Hydroxyl radical
$^{-}ONOO$	Peroxynitrite
ORAC	Oxygen radical antioxidant capacity
RDSC	$DPPH^{\bullet}$ scavenging capacity
TE	Trolox equivalents
TSPA	Total soluble phenolic acids
Trolox	6-hydroxy-2, 5, 7, 8- tetramethylchroman-2-carboxylic acid
WME	Williams' medium E growth medium
UV/Vis	Ultraviolet/Visible light

Introduction

Wheat (*Triticum aestivum* L.) is a major and important agricultural crop widely spread over the world. Most wheat cultivars cultivated today are categorized as common wheat and account for approximately 95% of the world's production. Growing evidence has showed that wheat and wheat-based products may have health beneficial properties. However, a few researches have focused on the potential of improving the nutritional or health promoting properties of wheat cultivars.

The goal of this research was to promote the potential use of selected wheat and wheat-based product to improve human health while enhancing food and agriculture economy. The specific objectives were:

1) To compare bran samples of different soft wheat cultivars for their phytochemical compositions, antioxidant properties, and antiproliferation activities.

2) To investigate the effect of genotype, growing environment and their interaction on the phytochemical compositions and antioxidant properties of soft wheat bran samples

3) To examine the differences in phytochemical contents, and antioxidant and antiproliferation properties in wheat bread processing fractions (upper crust, crumb, and dough).

Chapter 1: Literature Review

1.1 Introduction of Wheat

Common wheat (*Triticum aestivum* L.) is a major cereal crop consumed in many parts of the world, accounting for 30% of the total grain consumption with an annual production of over 660 million tons worldwide (FAO, 2010). The origins of wheat are thought to date back more than ten thousand years to the Levant region of the near east and Ethiopian highlands where remains of the wild progenitors of wheat have been discovered (Feldman & Kislev, 2007). Today, wheat is grown on the most land area of any commercial crop countries currently producing the most wheat include China, India, European countries, and the United States (Curtis, 2002; USDA, 2006).

Wheat contributes more calories than any other cereal crops (Adom & Liu, 2002; Shewry, 2009). It is nutritious, easy for transportation and storage, and can be processed into different types of food products. Wheat is considered as a good source of protein, minerals, B vitamins and dietary fiber although the environmental conditions can affect nutritional composition of wheat grains with its essential coating of bran, indicating that it is a great health-building food (Shewry, 2007). However, due to increased global competition, the US share of the wheat trade decreased and is estimated to increase slightly from 2006 to 2015 (USDA, 2006). The challenging situation for wheat in the US as of 2005 has generated considerable interests in research to improve the competitiveness and farm-gate (net) value of wheat (USDA, 2006).

Wheat is used in the production of many food products and mainly categorized into two quality classes: hard and soft according to its agronomic and end-use attributes which is based on its applicability for bakery type products. Gluten is the

main factor, determining the specific applications of wheat products due to its unique viscoelastic property (Pena, 2002). Hard wheat is known for their high gluten content, which is important in making breads and some types of wheat noodles. Soft wheat has low gluten protein content and is used for production of cookies, cakes, crackers, biscuits, pretzels, soup thickeners and batters (Briggle & Curtis, 1994; Hoseney, Wade, & Finley, 1988). In the US, common wheat is generally categorized into five classes: hard red winter wheat, hard red spring wheat, soft red winter wheat, soft white wheat, and hard white wheat - the newest class of wheat grown in the US according to their grain color, protein content, as well as growing season (Curtis, 2002; USDA, 2005). In addition, durum wheat (*Triticum durum*) which comprises less than five percent of global wheat production is also cultivated in the US because of its predominant uses in the pasta production (Curtis, 2002).

Conventional milling of wheat grains is based on separating the endosperm (which produces white flour when milled) from the bran layers and germ. Thus, the three main milling fractions of wheat kernel are endosperm, germ and bran. The endosperm is the largest part (83% by weight) of the kernel, and specialized for storing starch and proteins. Germ is the smallest part of wheat grain (3% by weight), which contains lipids, proteins and some bioactive components such as vitamins, phenolic acids, carotenoids, tocopherols, and phytosterols (Fulcher & Duke, 2002; Izydorzyk, Symons, & Dexter, 2002). Wheat bran is the outer layer of wheat kernel which consists of combined aleurone and pericarp, comprising around 15% of the kernel by weight. Aleurone is the most inner part of bran, consisting of a single layer of cells attached to the endosperm. Moving outwards the periphery of the wheat kernel are the nucellar, epidermis, testa, inner pericarp, and outer pericarp. Among all the layers of wheat kernel, aleurone might have the highest concentration of vitamins,

minerals, and bioactive phytochemicals such as tocopherols and phenolic acids (Fulcher & Duke, 2002). The high levels of bioactive compounds found in wheat bran and germ fractions have promoted the consumption of whole-wheat food products as opposed to refined wheat products which do not contain these bioactive rich wheat fractions.

1.2 Bioactive Compounds in Wheat Grain

It is known today that wheat naturally contains various classes of bioactive compounds, including phenolic acids, carotenoids, tocopherols, phytosterols, steryl ferulates, phytic acid, alkylresorcinols, plant lignans, and others (Adom & Liu, 2002; Adom, Sorrells, & Liu, 2003; Adom, Sorrells, & Liu, 2005; Crosby, 2005; Graf & Eaton, 1990; Kim, Tsao, Yang, & Cui, 2006; Li et al., 2005; Moore et al., 2005; Mattila, Pihlava, & Hellstrom, 2005; Panfili, Alessandra, & Irano, 2003; Zhou, Su, & Yu, 2004a; Zhou, Laux, & Yu, 2004b; Zhou, Yin, & Yu, 2005; Zhou & Yu, 2004c). Many of these bioactive compounds provide potential health-beneficial effects.

1.2.1. Phenolic Acids

Phenolic acids can be divided into two groups (Kim, Tsao, Yang, & Cui, 2006; Li, Shewry, & Ward, 2008; Ward et al., 2008), with hydroxybenzoic acid derivatives including vanillic, syringic, *p*-hydroxybenzoic, and gallic acids, and hydroxycinnamic acid derivatives including ferulic, *p*-coumaric, caffeic, and sinapic acids (Verma, Hucl, & Chibbar, 2009). They are thought to be one of the primary groups of compounds responsible for the total antioxidant and health promoting properties of wheat (Truswell, 2002; Slavin, Jacobs, & Marquart, 2000). Due to their varied roles in cell physiology, the quantity and quality of phenolic acids are different in grain tissues.

Phenolic acids are known to be concentrated primarily in the bran fraction of wheat, especially the aleurone layer. The content of phenolic acids in wheat flour is significantly lower than that in whole-grain flour (Beta, Nam, Dexter, & Sapirstein, 2005; Mattila, Pihlava, &, Hellstrom, 2005). Ferulic acid is the primary and most abundant phenolic acid in wheat grain. Smaller concentrations of *p*-hydroxybenzoic, vanillic, syringic, *o*-coumaric, *p*-coumaric, salicylic, sinapic acids are also observed in wheat (Kim, Tsao, Yang, & Cui, 2006; Liyana-Pathirana, Dexter, Shahidi, 2006a; Moore et al., 2005; Mpofu, Sapirstein, & Beta, 2006; Zhou, Laux, & Yu, 2004b).

The majority of wheat phenolic acids are present mainly in the bound forms, linked to cell wall structural components such as cellulose, lignin, and proteins through ester bonds (Parker, Hunter, & Spiegelman, 2005). The phenolic acids exist in wheat have three primary states, soluble free, soluble conjugated, and insoluble bound. A previous study by Moore et al. (2005) reported that the total phenolic content of soft wheat grains including soluble free, soluble conjugated, and insoluble bound fractions, ranged from 455 to 621 μg/g in wheat grain. This study also found the insoluble bound fraction to comprise the majority (91%) of wheat phenolics, with soluble conjugated and soluble free fractions comprising 8.7% and 0.58%, respectively (Moore et al., 2005).

1.2.2. Carotenoids

In cereals, color has been the most quality indicator provided by carotenoids. The yellow or brown pigments of wheat mainly derive from these carotenoids and their esters. On the other hand, carotenoids may play significant roles in the antioxidant capacities of wheat and wheat bran (Moore et al., 2005; Zhou, Laux, & Yu, 2004b). The most predominant carotenoids present in wheat grain are lutein and

zeaxanthin with concentrations of 0.5-1.44 μg/g and 0.2-0.39 μg/g grain respectively (Adom, Sorrells, & Liu, 2003; Humphries & Khachik, 2003; Moore et al., 2005). In addition, small contents of carotenoids including β-cryptoxanthin at 0.01-0.13 μg/g, and β-carotene at 0.09-0.21 μg/g were also detected in wheat grain. A report by Adom, Sorrells, & Liu (2003) evaluated carotenoid concentrations in different wheat fractions. They found 12-, 4-, and 2-fold higher concentrations of zeaxanthin, lutein, and β-cryptoxanthin respectively in the bran/germ fraction of wheat compared to endosperm, indicating that endosperm contain a low amounts of carotenoids, mostly concentrated in the germ and bran fractions. In addition, the same research group found that the bran/germ fractions contained four times more lutein, twelve times more zeaxanthin, and twice more β-cryptoxanthin than endosperm fractions (Adom, Sorrells, & Liu, 2005).

1.2.3. Tocopherols

The vitamin E includes four tocopherols and tocotrienols. Tocopherols have saturated phytyl side chains, while tocotrienols have isoprenyl side chain with three double bonds (Stone & Papas, 2003). Cereals are considered to be moderate sources of Vitamin E, total tocopherols and tocotrienols in wheat are in the range of 49-58 μg/g dry weight (Bock, 2000; Chung & Ohm, 2000; Stone & Papas, 2003). Previous studies have confirmed the presence of α-, β-, δ-, and γ-tocopherols in soft and hard wheat grain (Moore et al., 2005; Panfili et al., 2003), and previous studies reported that total tocopherols in wheat bran samples ranged from 3.96 to 12.71 μg/g (Zhou, Laux, & Yu, 2004b; Zhou, Yin, & Yu, 2005). In addition, soft and hard wheat samples contained approximately 10.0 μg/g of α-tocopherol which was the primary tocopherol in wheat grains according to the database from USDA (USDA, 2005).

Furthermore, the α-tocopherol concentration ranged from 1.28-21.29 μg/g in the eleven bran samples (Zhou, Laux, & Yu, 2004b; Zhou, Yin, & Yu, 2005).

Tocopherols are localized and concentrated in some parts of the kernel, either the germ or bran fraction. Thus, the content in the endosperm fraction is much lower than in the bran fraction. In addition, tocopherol concentrations of commercial wheat bran products were nearly two-fold higher than those in whole grains and four- or five-fold higher than those in refined flours (Holasova, 1997; Piironen et al., 1986).

1.2.4. Other Compounds

Wheat is generally recognized as a good source of several B vitamins (thiamine, niacin, Vitamin B_6 and folate). Endosperm provides only a small proportion of the thiamin and vitamin B_6 of the kernel, while wheat bran contains the majority of B vitamins (80% of the niacin, 32% of the thiamin, and 60% of the vitamin B_6), and a significant proportion of other vitamins (Pomeranz, 1988). Phytosterols are also highly retained in wheat kernel, especially in bran part, the total sterol contents of two wheat brans were 1.68 and 1.77 mg/g weight of bran with 4% ash content (Piironen, Toivo, & Lampi, 2002). The same research group also indicated that the higher the ash content, the higher was the sterol content. Steryl ferulates, the ferulic acid esters of plant sterols, and they have been detected in wheat bran in concentrations ranging from 0.3-0.4 mg/g (Hakala et al., 2002). In addition, phytic acid has been reported in wheat bran at 47.9 mg/g (Noort et al., 2010). Alkylresorcinols are compounds with long nonisoprenoid side chains attached to phenolic acids, which have been detected in wheat bran with total concentrations of 3.2 mg/g (Mattila, Pihlava, & Hellstrom, 2005). Lignans are one of the main groups of phytoestrogens in plant foods, which are localized in the fiber-containing aleurone and pericarp-testa cell walls, resulting in a

much higher levels of lignans in the bran fraction than in the wheat flour (Buri, Von Reding, & Gavin, 2004). Buri, Von Reding, & Gavin (2004) also reported that the aleurone layer of wheat has been shown to contain around 3-folds more secoisolariciresinol and matairesinol than wheat bran and about 10 times more than wheat grains. Furthermore, flavonoids, and especially anthocyanins, are the most important plant pigments for flower coloration producing bright colors, but only small amounts have been reported in hard spring wheat bran (Yeng, McDonald, & Vick, 1988). Although present in wheat, these micronutrients and phytochemicals are not thought to be the predominant compounds responsible for their antioxidant properties.

1.2.5. Total Phenolic Contents (TPC)

Although direct methods of quantification of the individual antioxidant in wheat are commonly used, indirect methods could also be carried out to determine the total concentration of certain antioxidant classes in wheat, such as total phenolic contents (TPC). Several studies have evaluated the TPC of wheat and its fractions using the Folin-Ciocalteu method. The TPC content of wheat grains was between 0.3 to 9.3 mg gallic acid equivalents (GAE)/g, while TPC contents of wheat bran, germ, and wheat-based products ranged from 0.2 to 6.1 mg GAE/g (Amarowicz & Maramac, 2002; Mpofu, Sapirstein, & Beta, 2006; Zhou, Su, & Yu, 2004a; Zhou & Yu, 2004c; Zhou, Yin, & Yu, 2005). Ferulic acid can also be used as a standard. The TPC values of wheat grain, endosperm, bran, and germ ranged from 0.2 to 12.2 mg ferulic acid equivalents (FAE)/g (Liyana-Pathirana & Shahidi, 2006b & 2006c; Velioglu, Mazza, Gao, & Oomah, 1998).

1.3 Health Benefits of Whole-Grain Consumption

Numerous epidemiological evidence has been accumulated to show that whole-grain intake may reduce the risk of certain chronic diseases, in particular cardiovascular disease (CVD), type 2 diabetes and certain cancers (de Munter et al., 2007; Fung, 2002; Koh-Banerjee et al., 2004; Mellen, Walsh, & Herrington, 2009; Sayhoun et al., 2006; Schatzkin et al., 2007; Seal, 2006). Although it might be the complex combination of components in the whole-grain matrix that may work together to give health benefits (Slavin, 2000; Fardet, 2010), tremendous animal and cohort studies have suggested that wheat bran is the key factor of wheat grain, may have a beneficial effect on the prevention of diseases, such as CVD and colorectal cancer (Alabaster, Tang, Frost, & Shivapurkar, 1993; Barbolt & Abraham, 1978; Barbolt & Abraham, 1980; Fardet, 2010; Jenab & Thompson, 1998; Jensen et al., 2004; Reddy et al., 1981; Reddy et al., 2000; Zile, Welsch, & Welsch, 1998).

Recent studies have disproved the role that dietary fiber only plays in prevention of chronic disease, indicating that other wheat bran components except for fiber might be responsible (Anderson, 2004; Park, Hunter, & Spiegelman, 2005). Since dietary antioxidants such as phenolic acids and tocopherols are known to be concentrated in bran fractions as compared to endosperm, they might be possible explanations for this relationship (Anderson, 2004; Fuchs et al., 1999; Slavin, 2004; Slavin, Jacobs, & Marquart, 2000). Saura-Calixto (2011) pointed out that dietary fiber and bound antioxidants (including phenolic acids and carotenoids) might be approached jointly in nutrition and health studies because antioxidants traverse the small intestine associated with dietary fiber as a carrier. A recent animal study by Carter, Madl, & Padula (2006) showed that the in-*vitro* antioxidant potential of wheat bran samples was correlated with their in-*vivo* anti-tumor activities. In addition, Zhou,

Laux, & Yu (2004b) reported that the antioxidant phytochemicals found in wheat bran fractions may modulate cellular oxidative status and prevent biologically important molecules such as DNA, proteins and membrane lipids from oxidative damage not only by reducing the availability of transition metals including Cu^{2+} that may act as catalyst to generate the first few free radicals that start the oxidative chain reaction, but also by reacting with and converting the peroxides to less reactive compounds, and consequently plays a role in reducing the risk of chronic diseases such as CVD and cancer. The phenolic antioxidants present in wheat bran have been shown to inhibit low-density lipoprotein (LDL) oxidation, possibly by binding with apolipoprotein-B and preventing the copper catalyst from binding to LDL (Liyana-Pathirana & Shahidi, 2007; Yu, Zhou, & Parry, 2005). Overall, the hypothesis is that antioxidants in wheat might be responsible for its health promoting properties has promoted further research in this area.

1.4 Reactive Species, Antioxidants, and Human Health

Reactive species (RS) include both reactive oxygen species (ROS) and reactive nitrogen species (RNS). The most prevalent ROS include peroxyl radicals ($ROO^{\bullet}$), superoxide anion radical ($O_2^{\bullet -}$), and hydroxyl radical ($^{\bullet}OH$)), while RNS generally include peroxynitrite ($^{-}ONOO$), nitric oxide ($^{\bullet}NO$) and nitrogen dioxide ($^{\bullet}NO_2$). The term free radical is often used, and normally means unpaired electrons in a molecule. However, RS also include highly reactive molecules, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), which are also considerably reactive and potentially detrimental to biological molecules, even though not technically free radicals.

RS not only can be generated as side products of general normal cellular metabolism, but also induced by the external sources. Some external sources of free

radicals are cigarette smoke, environmental pollutant, radiations, ultraviolet light, ozone, certain drugs, pesticides, anesthetics and industrial solvents (Kumar, 2011).

In human body, the oxidative status is controlled by an oxidative control system (Fridovich, 1998), which includes enzymes and cofactors. The major enzymes including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) are involved in the control of reactive species, and play an important role in detoxifying superoxide, hydrogen peroxide, and other lipid hydroperoxides. Be specifically, the SOD catalyzes the dismutation of reactive $O_2^{\bullet-}$ into oxygen and hydrogen peroxide. The catalase and GPx are responsible for converting the hydrogen peroxide into water. On the other hand, the cofactors for these enzymes including coenzyme Q_{10}, glutathione, ascorbic acid, α-tocopherol, as well as some metal ions such as selenium or copper ions may be also affecting the oxidative status (Diplock et al., 1998). These cofactors are tightly regulated in normal organism.

The oxidative stress reflects an imbalance between the production of RS and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Valko et al. (2007) also defined oxidative stress as “the harmful effect of free radicals causing potential biological damage”. The oxidative stress could result from low levels of antioxidants or overproduction of RS (Halliwell & Whiteman, 2004). RS are responsible for the oxidative damage of biological macromolecules such as DNA, lipids and proteins. Excessive amount of reactive species is thought to be involved in development of numerous diseases such as cancer, CVD, neurodegenerative disease, arthritis, and others (Diplock et al., 1998).

Antioxidants could be defined as substances that may protect your cells against the effects of free radicals which are molecules produced when your body breaks down food, or by environmental exposures like tobacco smoke and radiation. Free

radicals can damage cells, and may play a role in development of heart disease, cancer and other diseases.

Given the tremendous evidences linking oxidative stress and ROS to chronic conditions, there have been numerous research interests in the role of dietary antioxidants in preventing chronic diseases such as heart disease, CVD, and certain cancer (Barbaste et al., 2002; Temple, 2000; Willcox, Ash, & Catignani, 2004). One of the main forms of CVD is coronary heart disease (CHD), which has been associated with ROS and oxidative damage to arterial endothelial cells and circulating lipids. Oxidized LDL plays a key role in the development of atherosclerosis (Temple, 2000). Ingesting antioxidants and minimizing free radicals exposure may reduce LDL's contribution to atherosclerosis. Polyphenols, which are found in abundance in edible plants, are powerful in *vitro* antioxidants, and their consumption was shown to be inversely associated with morbidity and mortality from CHD (Virgili & Marino, 2008). Oxidized LDL cholesterol is preferentially taken up by macrophages to create the foam cells characteristic of fatty streaks, which are precursors of atherosclerotic plaques. Vitamin E and its combination with carotenoids or flavonoids can maintain oxidant/antioxidants balance and significantly protect LDL against oxidation, therefore reducing the risk of CVD (Barbaste et al., 2002; Willcox, Ash, & Catignani, 2004). In addition, a study of Khaw et al. (2001) found that an increase of 20 μmol/L in plasma ascorbic acid level was associated with a 21% reduction in cancer risk.

Nowadays, consumer's attentions have been more attracted by whole-wheat and wheat bran products for their potential health promotion and benefits. Ferulic acid, a predominant antioxidant found in wheat grain, has shown antiproliferative effect on HT-29 colon cancer cells (Ferguson, Zhu, & Harris, 2005). In addition, Yu, Zhou, & Parry (2005) found that the wheat bran extracts showed great inhibitory activities

against lipid oxidation in human LDL and free radical scavenging properties. US Dietary Guidelines also claimed that 3 to 10 servings of whole-grains intake per day should be recommended (USDA, 2005). So, it is necessary to generate renewed research interest in the development wheat-based food products which may include the bran fraction

1.5 Natural Antioxidants and Food Quality

Oxidation is responsible for a multitude of adverse effects and implications not only in human health, but as well as in food preservation and stability. Antioxidants can significantly prevent unwanted changes in flavor and nutritional quality of foods and have attracted much attention as food stabilizers. Both synthetic and natural antioxidants are widely used in food products. For instance, the addition of synthetic oxidation inhibitors into refined edible oils to improve their stability-related properties is a common practice. However, the reported deleterious effects on human health of these synthetic antioxidants such as butylated hydroxyanisole, or butylated hydroxytoluene have decreased their use and promoted the general consumers' acceptance of synthetic food additives (Kalantzakis & Blekas, 2006; Giron, Ruiz-Jimenez, & Luque de Castro, 2009). As a result, enrichment of edible oils with natural antioxidants to inhibit or suppress lipid oxidation becomes of great interest. These substances are relatively cheaper and do not produce any deleterious compounds under oxidation conditions (Veronica, Feliciano, Carlos, & Mara, 2011). Previous studies have appeared in the recent literature on the discovery and application of natural antioxidants (Shahidi & Zhong, 2010).

Some natural antioxidants, including vitamin E, soy protein isolates, cherry tissue, and olive extracts were reported to be effective against lipid oxidation, such as in meat products (Guntensperger, Hammerli-Meier, & Escher, 1998). Furthermore, previous studies showed that the extracts of wheat fractions and wheat-based food products suppressed lipid oxidation in olive and soy oils (Martinez-Tome et al., 2004). Be specifically, these natural antioxidants can inhibit and reduce lipid oxidation by scavenging free radicals including peroxyl and hydroxyl radicals, chelating metal ions, and quenching singlet oxygen, therefore reducing oxidation and extending shelf life of food products (Veronica, Feliciano, Carlos, & Mara, 2011). Some previous studies showed that wheat bran extract and wheat-based food exhibited radical scavenging capacities, and showed chelating activity against Fe^{2+} (Yu, Haley, Perret, & Harris, 2002). These studies suggested the potential uses of wheat antioxidants as a value-adding ingredient to prevent quality deterioration of food products and to maintain their nutritional value by food manufacturers (Fritsche & Johnston, 1998; Shahidi, 1997).

1.6 Bioaccessibility and Bioavailability of Dietary Antioxidants

Epidemiological studies have shown that bioactive compounds as dietary antioxidants in whole-wheat associated with reducing the risk of various chronic diseases (Mellen, Walsh, & Herrington, 2009; Schatzkin et al. 2007; Whent et al., 2012). Thus, a better understanding of the bioavailability of these dietary antioxidants of wheat becomes significantly important to us. Bioavailability can be defined as the extent to which a component in a food matrix can be absorbed and used by the body after ingestion (Anson et al., 2009a). Numerous studies involving phenolics and phenolic acids in wheat have investigated aspects of their bioavailability with *in-vitro*,

animal, and human studies. For example, Nystrom, Paasonen, Lampi, & Pirronen, (2007) found that the bound phenolic acids have very low bioavailability because wheat bran matrix extremely retards their access to the necessary enzymes (ferulate esterases and xylanases) in the human GI tract. In addition, the previous reported urinary recovery level of ferulic acid was as low as 3.1% in humans (Kern et al., 2003) and 3.9% in rats (Adom & Liu, 2002).

Various factors can influence the bioavailability of a compound, such as 1) bioaccessibility, 2) physical absorption, 3) tissue distribution, and 4) bioactivity (Stahl et al., 2002). Much is known about the absorption and tissue distribution, but only the studies relative to bioaccessibility work are discussed here due to this project. In a previous study, Anson et al. (2009a) pointed out that limited bioavailability of ferulic acid in cereal grain is influenced by its low bioaccessibility in the small intestine. This is because ferulic acid represents up to 90% of total phenolic acids in wheat and 99% of which in the insoluble bound form (Moore et al., 2005). In addition, Reboul et al. (2006) pointed out that there was a significant relationship between bioaccessibility ratios measured *in vitro* and the average bioavailability ratios measured in groups of healthy human. Furthermore, in another study of Anson et al. (2009b) showed that the bioaccessibility of ferulic, *p*-courmaric, and sinapic acids from the wheat bran is extremely low. They found that the most effective treatment was the combination of enzyme and fermentation that increased the bioaccessibility of ferulic acid from 1.1% to 5.5%. Due to cereal matrix, most of the insoluble bound phenolic compounds can't be attacked by enzymes in GI tract because their bioaccessibility, leading to low bioavailability (Wang, He, & Chen, 2014). Therefore, bioavailability of the bound phenolic compounds could be enhanced by increasing their bioaccessibility through

particle size reduction and suitable processing technologies, such as fermentation and thermal treatment.

1.7 Influences of Processing on Dietary Antioxidants in Wheat-Based Products

Wheat is the most important crop for bread-making because of its supreme baking performance in comparison with all other cereals. Due to tremendous use of wheat in bakery products, the potential processing steps of interest involved for wheat-based food products can include three major stages: mixing, fermentation and baking. It might be interesting to understand how the processing of wheat-based product could increase or decrease antioxidant properties and nutritious phytochemicals.

Generally, people have a common consensus about the degradation of dietary antioxidants when exposed to these processing. For example, Rodgers, et al. (1993) evaluated the stability of β-carotene during baking and pre-baking processing steps for yellow cake, sugar cookie, and bagels. This study found that baking caused significant reductions in the all-*trans* β-carotene isomer contents ranging from 74-85%. Ranhotra, Gelroth, Langemeier, & Rodgers (1995) found that baking of β-carotene fortified bread and cracker decreased total carotenoids from 4-23% with up to 20% of remaining carotenoids isomerized to 13- or 9-*cis* isomers. A recent study by Leenhardt et al. (2006) found the most significant decreases (66%) in total carotenoid contents occur during kneading with a high correlation of these losses to the lipoxygenase activity of wheat variety. This same study found less than 10% of losses in total carotenoids as a result of dough fermentation at 30 ºC, and losses during baking of 36-45%. In addition, the same group found significant decreases in the

tocopherol contents during bread making with 10-12% losses attributed to kneading, and 15-20% losses attributed to baking (Leehardt et al., 2006).

However, some other studies also reported that the fermentation and baking treatments did not cause any changes or even increased the dietary antioxidants such as phenolic acids in wheat-based food products (Abdel-Aal et al., 2013; Mattila, Pihlava, & Hellstrom, 2005). For example, Mattila, Pihlava, & Hellstrom (2005) reported that baking did not reduce the concentration of phenolic acids. A study by Moore, Luther, Cheng, & Yu (2009), indicated that generally, bran particle size and dough fermentation time had no effect on the antioxidant properties, however, increasing either baking temperature or heating time for the whole-wheat pizza crust may increase antioxidant activities. In addition, Abdel-Aal et al. (2013) studied on the effect of baking on free and bound phenolic acids in wholegrain bakery products. The results showed that baking increased the free phenolic acids, while bound phenolic acids decreased in bread products.

From these studies, it showed that wheat grain need to be processed for the production of bread and it is known that processing especially baking may influence the nutritional quality of bread by decreasing or increasing the levels of the bioactive compounds, and modify their bioavailability as well.

1.8 Chemical and Bioactive Analysis of Wheat

1.8.1. Identification and Quantification of Phytochemicals

The analytical methods used for determination of the phytochemicals in plant materials mostly depend on the resources available and the research objectives. They are usually classified as either measuring the total amounts of certain group of

compound, such as total phenolic content (TPC) and total anthocyanin content (TAC), or quantifying a specific phytochemical antioxidant by using HPLC or GC.

1.8.1.1 Total Phenolic Content

Folin-Ciocalteu method (FC) is an easy, rapid and economical method, which has been recognized as the most commonly applied method for TPC determination Singleton et al., 1999). The redox reagent (FC reagent) can react with phenolic compounds in plant extracts to form a blue complex that can be measured by visible spectrophotometer at 765 nm. The reducing power of sample solutions are thus compared against a standard phenolic acid, normally gallic acid, and results are expressed as mg gallic acid equivalents per unit of sample. However, some of previous studies showed that there was no significant correlation between TPC and phenolic compounds in plant materials analyzed by chromatographic methods (Atanackovic et al., 2012; Farvin and Jacobsen, 2013).

1.8.1.2 High Performance Liquid Chromatography (HPLC)

HPLC currently is the most popular and reliable technique for separation and quantification of individual compounds from the mixtures. The disadvantages of using HPLC as compared to other colorimetric technique include 1) high-purity solvents, 2) expensive operation system along with different types of detectors, 3) skilled persons needed for operating the system, 4) high-cost of columns. However, no other method is capable of providing more details about samples, particularly with the more advanced methods of detection, including mass spectrometry (MS) or nuclear magnetic resonance (NMR) detection. Thousands of studies utilized HPLC

for determination of phenolic compound from various plant materials have been reported in peer-reviewed publications (Ignat et al., 2011).

1.8.2. Antioxidant Property Assays

A number of *in vitro* methods have been developed and modified to measure the antioxidant activity through chemical assays in aqueous systems. These approaches have been applied to the estimation of antioxidant capacity by using free radical scavenging capacities. These assays are mainly divided into two categories: hydrogen atom transfer (HAT) reaction based and single electron transfer (ET) reaction based assays. In addition, it is suggested to use multiple antioxidant activity assays to evaluate a sample not only because antioxidant activity should not be concluded based on only one antioxidant assay, but also antioxidant assays vary in different mechanism so that it is difficult to compare one method with others (Badarinath et al., 2010).

1.8.2.1 Hydrogen atom transfer (HAT) reaction based assay

HAT-based assays apply a competitive reaction scheme, in which antioxidant and free radical generator compete for protecting or damaging fluorescent molecule thermally. The oxygen radical absorbance capacity (ORAC) and hydroxyl radical scavenging capacity (HOSC) are good examples of HAT-based assay.

The ORAC assay provides an indicator of a sample's ability to scavenge peroxyl radicals, and measures the oxidative degradation of the fluorescein (a fluorescent substance) after being mixed with free radical generators such as AAPH. AAPH is considered to produce the peroxyl radical by heating, which damages the fluorescent molecule, resulting in the loss of fluorescence. Antioxidants are

considered to prevent the fluorescent molecule from the oxidative degeneration. Decay curves (fluorescence intensity vs. time) are recorded and the areas between the two decays are calculated using Trolox as standard. However, some barriers are also subjected to this assay, such as 1) expensive equipment, 2) time-consuming, 3) only measure peroxyl radical.

HOSC assay is also a HAT-based assay to measuring the antioxidant capacities. The method is similar to ORAC by using competitive kinetics, but it varies in using different radical generator: ORAC uses AAPH to generate peroxyl radicals while HOSC assay uses a Fenton-like Fe^{3+}/H_2O_2 reaction to generate hydroxyl radicals (Moore et al, 2006). Like ORAC, HOSC only measure the pure hydroxyl radicals, and is performed on commonly available equipment, and uses inexpensive chemicals. An additional benefit is that HOSC is performed at a physiological pH. Meanwhile, the disadvantages of using HOSC include 1) can't measure lipophilic compounds, 2) carbon-center radicals can be formed to interfere with this assay. However, Cheng et al. (2007) has developed a method using ESR spin trapping detection and acetonitrile as the solvent for analysis of hydroxyl scavenging abilities on lipophilic antioxidants.

1.8.2.2 Single electron transfer (ET) reaction based assays

ET-based assays measure the capacity of an antioxidant to reduce an oxidant, which changes color when reduced. The degree of color change is correlated with the sample's antioxidant concentrations and activities. Most commonly used ET-based assay for antioxidant capacity evaluation is DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical scavenging assay.

The DPPH is a very stable radical with purple color and absorbance at 515 nm (Prior et al, 2005). The end point of this assay relies on the decreasing absorbance

when DPPH radical react with antioxidants through an electron transfer reaction, changing the color from purple into yellow (Magalhaes et al., 2008). The assay is easy to perform, partially because of the low-cost of the DPPH radical. However, the use of DPPH• scavenging assays has been questioned not only because the DPPH radical is sensitive to pH, but also the reaction was fairly slow and difficult to obtain absolute antioxidant values. An additional disadvantage is that DPPH radical is foreign to biological systems which make it difficult in *vivo* estimation.

The ABTS (2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate)) radical cation scavenging assay has also been called the TEAC (Trolox equivalent antioxidant capacity) assay, is another commonly used ET-based assay. The radical cation is an intense blue color, which disappears when the radical react with antioxidants. Absorbance is monitored at 734 nm (Magalhaes et al, 2008). ABTS radical could be generated in both chemical and enzymatic pathway. The assay is not only commonly used for hydrophilic antioxidants, but also very rapid and pH stable (Prior et al, 2005). However, like ABTS, its biological relevance is still unknown and questioned. Furthermore, there are a multitude of ET-based assays for measuring the reducing capacity of antioxidants. The assays are carried out at acidic (FRAP), neutral (TEAC), or basic (total phenols assay by FC reagent) conditions. The pH values have an important effect on the reducing capacity of antioxidants.

1.8.3. Cell-based Assays

It is very important to carry out cell-based assays after screening antioxidant capacity using *in vitro* chemical methods in order to approach some aspects of the bioavailability of the potential antioxidant such as uptake or partitioning in membranes, that are crucial to the effectiveness of the antioxidant *in vivo* (Lopez-

Alarcon & Denicola, 2013). The cell-based methods provide a quick measurement of bioactivity and are relatively cheaper than *in vivo* tests. However, cell-based assays are sometimes restricted by short of some important steps such as absorption, digestion, and metabolism of tested compounds before delivery to the cells. Therefore, cell-based assay may not provide a comprehensive understanding of the influences of exogenous antioxidants on oxidative stress *in vivo*.

1.8.3.1 Antiproliferative Assay

Antiproliferative assay is a simple and straightforward assay by exposing a cell culture to a treatment and then comparing cell growth to a control. It is a very commonly used *in vitro* assays for screening the antiproliferative activity of tested samples and their compounds. However, some barriers to this assay include long-time period for monitoring cell growth and limitation for numbers of samples that can be tested. Additional disadvantage is that the mechanism of antiproliferative activity is likely to depend on one component to the other, but this assay only focuses on which component is active but not to investigate its mechanism. This assay has received tremendous uses in previous studies, and their results were somehow compared to *in vivo* activity as reference (Wang et al., 2007).

1.8.3.2 Cellular Antioxidant Assay

Many intracellular radical probes have been reported in previous studies, and one of the most popular is the dichlorofluorescin diacetate (DCFH-DA) (Girard-Lalancette et al, 2009). Cellular antioxidant assay (CAA) is a cell-based assay for antioxidant activity evaluation in which DCFH-DA probe is used for the ROS level deduction. The non-polar probe DCFH-DA crosses the cell-membrane and enters the

cell, where its two acetates are cleaved down by esterases. The produced polar DCFH is trapped inside of the cell. Then, RS oxidizes DCFH into dichlorofluorescein (DCF), which fluoresces in response to the ROS level. Fluorescence is monitored to determine the levels of RS in cells (excitation wavelength = 485 nm; emission wavelength =530 nm).

Furthermore, there are some disadvantages that influence the uses of the CAA. Firstly, except for common reactive species O_2•- and H_2O_2, other reactive species might be capable for oxidizing DCFH into DCF (Wardman, 2008). Secondly, different concentrations of antioxidant or probe used for CAA might affect the accuracy of final result. Additionally, natural light may induce photo-oxidation to interfere with results by increasing background fluorescence so that experiments should be strictly conducted in the dark or under yellow light (Soh, 2006; Wardman, 2008).

Chapter 2: Phytochemical compositions and antiproliferative activities of bran fraction of ten Maryland-grown soft winter wheat cultivars

2.1 Abstract

Phytochemical (ferulic acid, tocopherols, and carotenoids) composition and antiproliferative activities of bran samples of the ten soft winter wheat cultivars grown in Maryland were investigated. All extracts were assayed for total phenolic content and free radical scavenging capacities by multiple colorimetric assays along with cellular antioxidant activity (CAA) and antiproliferative activity. Ferulic acid was the predominant phenolic acid in all ten wheat bran samples with concentration ranging between 1.1 to 1.7 mg/g. The concentrations of lutein, zeaxanthin, and β-carotene ranged between 1.0-1.4, 0.2-0.3, and 0.1-0.2 µg/g, respectively. Significant amount of *α*-tocopherol (2.3 to 5.3 µg/g) was quantified in all bran samples along with minor quantity of *δ*-tocopherol (~ 0.1 µg/g). No significant correlation between ferulic acid, tocopherol and carotenoid content and in vitro antioxidant radical scavenging capacity or total phenolic content was observed. The Jamestown wheat bran demonstrated significant antiproliferative activities against both HT-29 and Caco-2 colon cancer cells at concentration of 50 mg bran equivalent (BE)/mL. The present research necessitates further careful investigation regarding health beneficial effects claims using just different antioxidant assays.

2.2 Introduction

Wheat and its products are important part of human diet. Wheat is the third important field crops in both planted acreage and gross farm receipts, behind corn and soybeans in US (USDA, 2012). It is one of most important commodity agricultural product consumed globally. Wheat bran, a byproduct of flour milling industry is an important, cheap, and readily available source of dietary fiber. It was primarily used as animal feed, however, in recent years; wheat and other cereals bran have gained importance in various food product formulations (Vetter, 1988). This has been attributed to the recent epidemiological studies with whole grain foods which suggest that whole grains provide health promoting protective effects against certain types of cancers, cardiovascular diseases and type-2-diabetes (Astorg et al., 2002; Willcox, Ash, & Catignani, 2004; Zhou, Su, & Yu, 2004a). Most of the health beneficial effects of the whole grains have been due to bioactive phytochemicals, vitamins, minerals, and fiber present in high concentration in bran fraction of the grain.

The bran fraction constitutes approximately 15-20% of dry grain weight. It usually comprises of outermost portion of the grain composed of several layers (pericarp, testa, and hyaline) that are characterized by distinct structures and composition. The inner layer is composed of aleurone cells and it constitutes approximately 6-7% of the bran. The percent values for bran fractions vary with the type of wheat cultivar (Hemery et al., 2010).

There have been large numbers of peer reviewed publications on antioxidant activity of wheat bran fraction in recent years. Significant antioxidant activity and phenolic compounds have been detected in wheat, wheat bran and wheat based products (Liyana-Pathirana & Shahidi, 2007; Moore et al., 2005; Moore, Liu, Zhou, &

Yu, 2006a; Zhou, Laux, & Yu, 2004b; Zhou, Su, & Yu, 2004a; Zhou, Yin, & Yu, 2005). The wide variations in the reported antioxidant activity values stems from the differences in procedures used for the assay of antioxidants and the methodologies used for extraction of antioxidants (Luthria, 2006).

In previous publications by Zhou, Su, & Yu (2004a), the authors described phytochemicals and antioxidant properties of seven wheat cultivars from four countries. In other study the same group carried out antioxidant activity and phytochemical analysis of hard red winter wheat cultivars (Zhou, Laux, & Yu, 2004b). In a very recently published study, authors reported phytochemicals composition, antioxidant activities, and antipoliferative activities of ten wheat flour samples (Lv et al., 2012). In continuation of our research on wheat, we report here a systematic comparison of three classes of phytochemicals (phenolic acids, tocopherols, and carotenoids from bran fraction of ten soft red winter wheat cultivars commonly cultivated in the mid-Atlantic region of the United States. In this study, we also examined the scavenging activities against hydroxyl ($HO^{\bullet}$), 2, 2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), 2, 2'azinobi-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{\bullet+}$), and peroxyl radicals along with total phenolic content by commonly used Folin-Ciocalteu assay. In addition, reduction of oxidative stress in human liver cancer Hep G2/C3A cells, and antiproliferative activities in HT-29 and Caco-2 human colon cancer cells of soft winter wheat bran samples were also investigated. Results from this study warrant further research into the industrial utilization of bran fractions of Maryland-grown soft winter wheat cultivars and careful investigation regarding health beneficial effects claims using different radical scavenging activities assays is also needed.

2.3 Materials and Methods

2.3.1. Wheat Samples

Ten soft red winter wheat (*Triticum aestivum* L.) cultivars, SS520, SSMPV57, SS5205, USG3555, USG3665, USG3315, Branson, Shirley, Jamestown and Chesapeake, representing a sample of elite commercial cultivars currently grown in the mid-Atlantic, were grown in the field at Clarksville (MD) in yield trial plots 4 m long by 1m wide at a density of approximately 350,000 plants/ha. Plots were planted following a crop of corn on October 2010. Plots were fertilized with a fall application of 16 kg/ha of nitrogen, 40 kg/ha of phosphorus and 80 kg/ha of potassium. Additionally 30-80 kg/ha of nitrogen was applied in March or April 2011. Grain from the field plots was mechanically harvested, threshed and cleaned of debris prior to laboratory testing.

2.3.2. Chemicals and Reagents

Disodium ethylenediaminetetraacetate (EDTA), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$), fluorescein (FL), lauryl sulfate sodium salt, sodium hydroxide, ethyl ether, ethyl acetate, 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox), tocopherols (α-, δ-, and γ-), ascorbic acid, β-carotene, 2',7'-dichlorofluorescein diacetate (DCFHDA), fetal bovine serum, hepes (pH 7.4), L-glutamine, insulin, hydrocortisone, antibiotic-antimycotic and gentamicin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Iron (III) chloride, ABTS^{TM} chromophore, diammonium salt, and thirty percent ACS-grade hydrogen peroxide were obtained from Fisher Scientific (Fair Lawn, NJ, USA). 2, 2$^{'}$-azinobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals

(Richmond, VA, USA). Ultrapure water was used for all experiments, which was prepared by an ELGA Purelab ultra Genetic polishing system with < 5 ppb TOC and resistivity of 18.2 mΩ (Lowell, MA, USA). Human hepatoma cell line Hep G2/C3A, human colorectal cell lines HT-29 and Caco-2 were obtained from American Type Culture Collection (ATCC). All cell culture media components were purchased from Invitrogen (Grand Island, NY, USA). All other chemicals and solvents were of the analytical grade and were used directly without further purification.

2.3.3. Preparation and Extraction of Wheat Bran

Each wheat sample was ground to a particle size of 40-mesh using a handheld coffee grinder and separated into flour and bran fraction. The bran yield was about 17.0-22.2 %. The milled bran samples were kept in a -20 ^{o}C freezer in airtight containers until analysis. The extraction of antioxidants assay was conducted according to a previously reported laboratory procedure (Moore, Liu, Zhou, & Yu, 2006a). Half gram of ground wheat bran was extracted with 5 mL of 50% acetone for 24 hours under nitrogen at ambient temperature. The acetone extracts were used for estimating total phenolic content (TPC), relative DPPH$^{\bullet}$ scavenging capacity (RDSC), oxygen radical absorbing capacity (ORAC), hydroxyl radical scavenging capacity (HOSC), and ABTS$^{\bullet+}$ scavenging capacity. The extracts were stored under nitrogen in dark at low temperature until further analysis.

2.3.4. Total Phenolic Content (TPC) in Wheat Bran

The TPC of wheat bran was determined according to a laboratory procedure described previously (Yu et al., 2002). In general, the final reaction mixture contained 50 µL wheat bran extract, 250 µL of the Folin–Ciocalteu reagent, 750 µL of 20%

sodium carbonate, and 3 mL ultrapure water. Gallic acid was used as the standard. After 2 hours of reaction at ambient temperature in dark, absorbance was read at 765 nm. Results were expressed as mg of gallic acid equivalent (GAE) per gram of wheat bran on a dry weight basis.

2.3.5. Ferulic Acid in Wheat Bran

Each wheat bran sample was analyzed for its ferulic acid soluble free and conjugated and insoluble bound according to the laboratory method described by Moore et al. (2005). Ground wheat bran was extracted with acetone/methanol/water (7:7:6, v/v/v) first to obtain the soluble supernatant and residue. The residue was hydrolyzed with sodium hydroxide, and then extracted with ethyl ether and ethyl acetate (1:1, v/v) for analysis of insoluble bound ferulic acid. Soluble, free, and conjugated ferulic acid in the supernatant was separated under acidic conditions (pH = 2). Ferulic acid was extracted with ethyl ether and ethyl acetate (1:1, v/v). After evaporating the organic phase under nitrogen, residue was re-dissolved in methanol and filtered through a 0.45 µm membrane filter. The filtered extract was analyzed for ferulic acid quantification by the HPLC analysis. Briefly, the elution program was as follows: mobile phase A consisted of acetic acid/H_2O (2:98, v/v) and mobile phase B consisted of acetic acid/acetonitrile/H_2O (2:30:68, v/v/v). Elution was programmed from 10 to 100% B in 42 min with a flow rate of 1.0 mL/min. Injection volume was 10 µL. The ferulic acid was identified through comparing to the standard for retention time. Quantification was based on the area under the peak of external standards. Results were expressed as µg/g of wheat bran on a dry weight basis.

2.3.6. Carotenoid Composition

Two hundred mg of ground wheat bran was extracted with 10 mL of methanol/tetrahydrofuran (1:1, v/v) for 15 hours at ambient temperature. The resulting extraction mixture was centrifuged at 2,000 rpm. The solvent was removed from the supernatants under N_2. The residues were re-dissolved in 2 mL of methanol/acetonitrile/iso-propanol (54:44:2, v/v/v), filtered through a 0.45 μm membrane filter, and analyzed for carotenoids using a HPLC. HPLC separation was accomplished using a Shimadzu LC-20AD with an autosampler, a Phenomenex C18 column (4.6 mm i.d. × 250 mm, 5 μm particle size) at 25 ^{o}C and a UV-VIS detector at 450 nm, according to a previously described laboratory protocol (Moore et al., 2005). Water as solvent A and methanol/acetonitrile/iso-propanol (54:44:2, v/v/v) as solvent B. The gradient procedure was as follows: 1) the gradient was linear from 95% to 99% of solvent B, and the flow rate was 1 mL/min in the first 10 min, 2) 99% of solvent B and a flow rate of 1 mL/min for 10 min, and 3) the gradient was linear from 99% to 95% of solvent B for the last 5 min. Twenty μL of each standard or sample was injected. A standard curve was developed from the known standards, and peak area was used for quantification. Results were expressed as μg/g of wheat bran on a dry weight basis.

2.3.7. Tocopherol Content

HPLC separation was accomplished using a Shimadzu LC-20AD with an autosampler and an UV-VIS detector, and a Phenomenex C18 column (4.6 mm i.d. × 250 mm, 5 μm particle size) at 25 $^{\circ}$C according to a previously described protocol. (Zhou, Yin, & Yu, 2005) The tocopherols were separated using an isocratic elution

with a mobile phase 1% solvent A (water) and 99% solvent B (acetonitrile). Flow rate was 1.5 mL/min. Injection volume was 20 µL for each standard or sample. A standard curve was developed from the known standards (α-, δ-, and γ-tocopherol), and peak areas were used for quantification. Results are expressed as µg/g of bran on a dry weight basis.

2.3.8. Measurements of Radicals Scavenging Capacity

The radical scavenging capacity was carried out by four different assays.

2.3.8.1 Relative DPPH$^{\bullet}$ Radical Scavenging Capacity (RDSC)

The RDSC values were determined according to a previously described laboratory procedure (Cheng, Moore, & Yu, 2006). Briefly, the final reaction mixture contained 100 µL bran extract, Trolox standard or solvent (positive and negative controls), and 100 µL of 0.2 mM working DPPH$^{\bullet}$ solution. The absorbance was measured at 515 nm every minute for 40 min, using a Victor^3 multi-label plate reader (PerkinElmer, Turku, Finland). Trolox was used as the antioxidant standard. The RDSC was calculated from the area under the curve and expressed as µmol of Trolox equivalents (TE)/g of wheat bran sample.

2.3.8.2. Oxygen Radical Absorbance Capacity (ORAC)

The ORAC values were determined following a previously reported laboratory protocol (Moore et al., 2005). FL was used as the fluorescent probe and a Victor^3 multilabel plate reader (Perkin-Elmer, Turku, Finland) was used to measure fluorescence. Briefly, the initial reaction mixture contained 225 µL of 8.16×10^{-8} M

FL, and 30 µL bran sample, blank or standard, were preheated in the plate reader at 37 °C for 20 min. Then 25 µL of 0.36 M AAPH was added to each well and the fluorescent of the mixture was recorded every 2 min over 2 hours at 37 °C. Trolox was used as a standard. The results were expressed as µmol TE per gram of wheat bran on a dry weight basis.

2.3.8.3. Hydroxyl Radical (HO•) Scavenging Capacity (HOSC)

The HOSC assay was performed according to a previously reported laboratory procedure using a Victor^3 multi-label plate reader (PerkinElmer, Turku, Finland) (Moore, Yin, & Yu, 2006b). In brief, the reaction mixture consisted of 170 µL of 9.28 × 10^{-8} M fluorescein (FL), 30 µL bran sample, blank, or Trolox standard, 40 µL of freshly prepared 0.1990 M H_2O_2 and 60 µL of 3.43 mM $FeCl_3$. The fluorescence was recorded every 4 min for 4 hours. The HOSC was quantified using the area under the curve and expressed as µmol TE/g bran on a dry weight basis.

2.3.8.4. $ABTS^{\bullet+}$ Scavenging Capacity

The scavenging ability against $ABTS^{\bullet+}$ of wheat bran extract was measured using a previously reported protocol (Zhou, Laux, & Yu, 2004b). $ABTS^{\bullet+}$ radicals were generated by oxidizing a 5 mM aqueous solution ABTS with manganese dioxide under ambient temperature for 30 min. The final reaction mixture contained 1.0 mL $ABTS^{\bullet+}$ solution with an absorbance of 0.700 ± 0.005 at 734 nm and 80 µL of 50% acetone for the control or 80 µL of the wheat bran sample or standard solution. The absorbance was read at 734 nm after 90 s of reaction. Trolox was used as a standard.

Results were calculated using a standard curve and expressed as µmol TE/g of wheat bran on a dry weight basis.

2.3.9. Cellular Antioxidant Assay (CAA) of Wheat Bran Extracts

2.3.9.1. Extract Preparation and Cell Culture

All ten wheat bran samples were chosen for their cellular antioxidant activity study. Each wheat sample (0.45 g) was extracted with 4.5 mL 50% acetone overnight at ambient temperature. The supernatant was collected, and acetone and water in the supernatant were evaporated using a nitrogen evaporator. The solid residues were re-dissolved in DMSO. Hep G2/C3A cells were plated at 6×10^4 cells per well of culture medium in 96-well plates and kept in a 37 °C atmosphere with 5% carbon dioxide. Culture medium for Hep G2/C3A cells consisted of the complete Williams' medium E growth medium (WME), 5% FBS, 2 mM L-glutamine, 10 mM hepes, 5 µg/mL insulin, 0.05 µg/mL hydrocortisone, 50 units/mL antibiotic/antimycotic, and 100 µg/mL gentamicin.

2.3.9.2. Determination of Cytotoxicity

Cytotoxicity of the ten wheat bran extracts were determined by a luciferase enzyme method using the ATP lite reagent kit (Perkin-Elmer Life and Analytical Sciences, Shelton, CT, USA). Luminescence was measured using the Victor^3 multi-well plate reader and relative luminescence values are proportionate to the number of living cells (Perkin-Elmer, Turku, Finland). Hep G2/C3A (6×10^4 cells/well) was seeded using the culture medium for 24 hours before treatment. A luminescence

reading was taken on the cells 24 hours post-initial treatment. Concentrations of bran extracts that had >10% decrease in luminance compared to the control were considered as cytotoxic. Vehicle medium for Hep G2/C3A contained 1% DMSO.

2.3.9.3. Cellular Antioxidant Assay (CAA) Test

In vivo antioxidant capacities of wheat bran samples were determined by CAA assay owing to previous study (Wolfe & Liu, 2007). 6×10^4 cells/well of Hep G2/C3A cells were seeded in 96-well-plate in the culture medium and kept in a 37 ℃ atmosphere with 5% carbon dioxide. Treatment media was added to pretreat the cells for 24 hours, then, media was removed and cells were washed with warm HBSS. Hep G2/C3A cells were then cultured with antioxidant media, the Williams' E medium with added 10 mM Hepes, 2 mM L-glutamine and bran extracts, and 25 µM DCFHDA for another 1 hour. Then, 100 µL of 600 µM ABAP was added into the cultures and reading was taken immediately. Fluorescence was read in 5-minute intervals for one hour. The plate reader was set at 37 ℃ with an emission wavelength of 538 nm and excitation wavelength of 485 nm. Gallic acid was used as an antioxidant standard. The results were expressed as mg of GAE per gram of bran samples.

2.3.10. Antiproliferative Effects of Different Wheat Extracts on HT-29 and Caco-2 Human Colon Cancer Cells

The wheat bran extracts with stronger antioxidant activities were tested for their antiproliferative effects in HT-29 and Caco-2 human colon adenocarcinoma cells according to the method described by Slavin, Kenworthy, & Yu (2009). Culture

medium consisted of McCoy's 5A media supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic.

Cells were plated at 2.5×10^3 cells/well culture medium in 96-well plates. After a 24-hour incubation time, the culture medium was replaced with 100 µL of treatment media containing 9.0, 22.5, 45.0 mg of bran equivalents (BE)/mL of treatment media. All media had a final concentration of 1% DMSO (v/v). Cell proliferation was studied using the ATP-Lite 1 step kit (Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA). Luminescence readings were taken on a Victor3 multi-well plate reader (Perkin Elmer, Turku, Finland) immediately prior to treatment and at 24, 48, 72, and 96 hours of treatment. A separate plate was used for each reading. Treatment and control media were replaced every 24 hours until a reading was taken on that plate.

2.3.11. Statistical Analysis

Data was reported as mean ± standard deviation (n = 3). Differences between means were determined by analysis of variance (ANOVA) with Tukey's HSD post hoc test, which were analyzed with SPSS (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL, USA). Correlation analyses were performed using a two-tailed Pearson's correlation test. Statistical significant was declared at $P < 0.05$.

2.4. Results and Discussion

2.4.1. Phytochemical Composition of Wheat Bran

2.4.1.1. Ferulic Acid

Table 2.1. Ferulic acid content of bran samples of 10 Maryland-grown soft winter wheat cultivars [a]

	Insoluble bound (µg/g)	Soluble (µg/g)	Total (µg/g)
SS 520	1184.29a ± 235.58	5.23a ± 1.34	1189.5a ± 234.7
SS MPV57	1246.25a ± 210.81	4.48a ± 0.97	1250.7a ± 210.9
SS5205	1381.73a ± 148.81	6.20ab ± 1.40	1387.9a ± 148.2
USG 3555	1725.32b ± 198.53	5.62ab ± 1.14	1730.9b ± 198.0
USG 3665	1577.84ab ± 88.60	7.02b ± 2.00	1584.9ab ± 87.6
USG 3315	1635.11b ± 164.68	6.95b ± 1.75	1642.1b ± 166.2
Branson	1497.76ab ± 99.36	5.07a ± 0.71	1502.8ab ± 99.2
Shirley	1402.56a ± 249.07	5.04a ± 1.30	1407.6a ± 248.6
Jamestown	1279.26a ± 126.76	6.15ab ± 1.28	1285.4a ± 127.0
Chesapeake	1531.97ab ± 99.81	4.85a ± 1.18	1536.8ab ± 99.7

[a] SS520, SSMPV57, SS5205, USG3555, USG3665, USG3315, Branson, Shirley, Jamestown and Chesapeake are ten soft winter wheat cultivars. Data are expressed as the mean ± standard deviation (n = 3).

Ferulic acid was the predominant phenolic acid identified in all bran fractions. Similar results were reported in previous studies on wheat bran (Mateo Anson et al., 2008; Zhou, Su, & Yu, 2004a). The concentration of total soluble (free and conjugated) ferulic acid ranged from 4.48 µg/g in SS MPV57 wheat bran to 7.02 µg/g in USG 3665 wheat bran sample (**Table 2.1**). The concentration of total soluble (free and conjugated) ferulic acid in all samples was less than 1%. Over 99% of the ferulic acid was identified as insoluble bound form. Insoluble bound ferulic acid concentration ranged from 1184.29 µg/g in SS520 wheat bran to 1725.32 µg/g in USG 3555 wheat bran sample. Similar high concentration (89.2 to 94.6%) of

insoluble bound form of ferulic acid has been reported in previous study by Moore et al. (2005).

The concentration range of total ferulic acid (1157.8-1730.9 µg/g) in the bran fraction was 5 times greater than (172.9–297.6 µg/g) for soft winter wheat flour (Lv et al., 2012) and 98.54-230.50 µg/g for wheat bran samples of collected from four countries as reported by Zhou, Su, & Yu (2004a) (**Table 2.1**).

2.4.1.2. Carotenoid Profile

Lutein was found to be the predominant carotenoid in all wheat bran samples. Lutein's concentration ranged from 0.96 to1.37 µg/g. This was comparable with the previously reported (Zhou, Su, & Yu, 2004a) where concentration of lutein ranged from 0.50-1.80 µg/g in bran samples of the seven wheat cultivars collected from four countries. The values reported for lutein in the bran fraction were higher than those reported in the soft winter wheat flour (0.27–0.46 µg/g) of the same wheat cultivar (Lv et al., 2012) (**Table 2.2**). Similarly, the zeaxanthin levels in the current study which ranged from 0.21 to 0.31 µg/g were comparable to the previously reported values (0.25 to 0.40 µg/g) by Zhou, Su, & Yu (2004a). These values were consistently higher than those reported in soft winter wheat flour (Lv et al., 2012). All tested soft wheat bran samples contained β-carotene, between 0.09-0.15 µg/g (**Table 2.2**). However, no β-carotene was detected in soft red wheat bran from Illinois as well as Burlington wheat bran from Colorado (Zhou, Su, & Yu, 2004a). Total carotenoids content in soft winter wheat brans ranged between 0.23 to 0.32 µmol/100 g with lowest yield in Jamestown cultivar and highest yield obtained from USG3315, respectively (**Table 2.2**). This range was comparable to 0.20-0.33 µmol/100 g with

the bran samples of two hard winter wheat cultivars grown in two Colorado locations (Zhou, Yin, & Yu, 2005), and the 0.12-0.68 µmol/100 g bran samples of the seven wheat cultivars collected from four countries (Zhou, Su, & Yu, 2004a).

2.4.1.3. Contents of Tocopherol

α-Tocopherol was identified as a predominant tocopherol in all ten wheat brans investigated in the present study. The concentration of α-tocopherol ranged from 5.26 to 7.60 µg/g **(Table 2.2)**. Maximum amount of α-tocopherol was extracted from Shirley bran sample and minimum quantity was quantified in USG3555. The concentration of α-tocopherol varied between 4.10 to 6.51 µg/g. These are in agreement with the previously reported values by Zhou, Yin, & Yu (2005) from bran samples of two hard red winter wheat cultivars grown in two Colorado locations. However, the concentration was 10-fold greater than those recently reported for soft winter wheat flour (0.30–0.59 µg/g) by Lv et al. (2012). In addition, we also identified δ-tocopherol in all bran samples with concentration ranging from 0.12-0.18 µg/g, agreeing with the levels reported in the previous study of Zhou, Yin, & Yu (2005). In addition, the contents of total tocopherols content (1.26-1.80 µmol/100 g bran) in our tested bran samples were consistent to the previously reported values (1.87-2.63 µmol/100 g) for bran samples of two hard winter wheat cultivars (Zhou, Yin, &Yu, 2005), and 0.92-6.90 µmol/100 g bran detected in the seven wheat bran samples from four countries (Zhou, Su, & Yu, 2004a). These results suggest that selected Maryland-grown soft winter wheat bran may serve dietary source of α-, and δ-tocopherols.

Table 2.2. Carotenoid and Tocopherol profiles of bran samples of 10 Maryland-grown soft winter wheat cultivars [a]

	Lutein (µg/g)	Zeaxanthin (µg/g)	β -Carotene (µg/g)	Total Caros (µmol/100 g)	α-Tocopherol (µg/g)	δ-Tocopherol (µg/g)	Total Tocos (µmol/100 g)
SS520	1.26ab ± 0.17	0.29ab ± 0.03	0.12a ± 0.08	0.29ab ± 0.04	6.03ab ± 2.13	0.12a ± 0.03	1.43ab ± 0.50
SSMPV57	1.35b ± 0.18	0.30ab ± 0.05	0.09a ± 0.06	0.31b ± 0.05	6.30ab ± 1.73	0.16a ± 0.05	1.50ab ± 0.41
SS5205	1.14ab ± 0.25	0.27ab ± 0.07	0.14a ± 0.05	0.27ab ± 0.06	6.32ab ± 1.29	0.16a ± 0.06	1.50ab ± 0.39
USG3555	1.28ab ± 0.26	0.30ab ± 0.07	0.12a ± 0.08	0.30ab ± 0.06	5.26a ± 1.19	0.16a ± 0.04	1.26a ± 0.29
USG3665	1.08ab ± 0.26	0.25ab ± 0.07	0.11a ± 0.09	0.25ab ± 0.07	6.00ab ± 1.17	0.18a ± 0.06	1.43ab ± 0.29
USG3315	1.37b ± 0.37	0.31b ± 0.07	0.12a ± 0.02	0.32b ± 0.07	7.31ab ± 1.90	0.18a ± 0.07	1.74ab ± 0.46
Branson	1.19ab ± 0.29	0.26ab ± 0.06	0.15a ± 0.06	0.28ab ± 0.06	6.79ab ± 1.46	0.15a ± 0.06	1.61ab ± 0.35
Shirley	1.23ab ± 0.22	0.29ab ± 0.06	0.10a ± 0.06	0.29ab ± 0.05	7.60b ± 1.83	0.14a ± 0.04	1.80b ± 0.43
Jamestown	0.96a ± 0.36	0.21a ± 0.08	0.13a ± 0.10	0.23a ± 0.09	6.39ab ± 1.86	0.14a ± 0.06	1.52ab ± 0.45
Chesapeake	1.19ab ± 0.34	0.27ab ± 0.08	0.13a ± 0.07	0.28ab ± 0.07	7.29ab ± 2.11	0.17a ± 0.06	1.73ab ± 0.50

[a] SS520, SSMPV57, SS5205, USG3555, USG3665, USG3315, Branson, Shirley, Jamestown and Chesapeake are ten soft winter wheat cultivars. Total Caros, total carotenoids; Total Tocos, total tocopherols. Data are expressed as the mean ± SD (n = 3). Means marked with the same letter are not significantly different ($P > 0.05$).

2.4.2. TPC

The SS3555 wheat bran contained the highest total phenolic content (TPC) of 2.43 mg GAE/g bran, whereas the lowest TPC of 2.02 mg GAE/g bran was observed in the SS5205 wheat bran. There was no significant difference in TPC in different bran samples of ten soft winter wheat cultivars. The value for the TPC was higher than the soft winter wheat flour (1.66 to 2.01 mg of GAE/g) values reported by Lv et al. (2012), but lower (2.7-3.5 mg GAE/g) than the bran samples from hard winter wheat cultivars (Moore, Liu, Zhou, & Yu, 2006a). There was no correlation between the total phenolic content as determined by Folin-Ciocalteu and the predominant ferulic acid content quantified and identified by in all bran samples HPLC analysis (r = 0.125, $P > 0.05$) (data not shown). The variation between TPC and HPLC analysis may either be attributed to other phenolic compounds that were not characterized in the present study or due to other potential inferences associated with the colorimetric Folin-Ciocalteu assay. Thus, one needs to be cautious when using only total phenolic content determination by colorimetric Folin-Ciocalteu assay.

2.4.3. Radical Scavenging Capacities

2.4.3.1. RDSC

The SS3665 wheat bran had the highest RDSC of 3.20 μmol TE/g, and the lowest RDSC of 2.18 μmol TE/g was observed in the Chesapeake wheat bran (**Table 2.3**). There was no significant difference ($P > 0.05$) among RDSC values for ten soft winter wheat bran fractions investigated in the present study. In the previously reported study by Cheng, Moore, & Yu (2006), $DPPH^{\bullet}$ scavenging capacity was determined on wheat samples, but the methodology and reporting units were different from the current study, direct comparison between the results was not possible.

Similar limitation associated between different antioxidant activities with varying procedures was cited in a recent study (Finley et al., 2011). In addition, the Pearson correlation test showed that RDSC values were marginally correlated with the tocopherols (r = -0.597, P < 0.001) and TPC (r = 0.623, P < 0.001) under the current experimental conditions. The values reported for the DPPH$^{\bullet}$ scavenging activity was 3-fold greater than the soft winter wheat flour (Lv et al., 2012). This variation in the values may be attributed to high phytochemicals (ferulic acid, tocopherols, and carotenoids) concentrations in the bran sample as compared to the wheat flour. No distinct correlations between RDSC and other antioxidant assays namely ABTS, HOSC and ORAC were observed in the current study.

2.4.3.2. ORAC

The ORAC value of the ten soft winter wheat bran ranged between 39.91 to 61.50 μmol TE/g with Shirley cultivar showing the lowest and USG3555 wheat bran the maximum ORAC value (**Table 2.3**). Our current results were similar to the values previously reported for the bran samples from the two Colorado locations (Zhou, Yin, & Yu, 2005). However, the present results were significantly greater than the values reported in a recent study by Lv et al. (2012) for soft winter wheat flour (29.90 to 40.20 μmol TE/g). There were no correlations between ORAC and other radical scavenging capacity assays namely, ABTS, HOSC and RDSC.

2.4.3.3. HOSC

All tested wheat bran demonstrated hydroxyl radical scavenging capacity (**Table 2.3**). The USG3665 wheat bran had the highest HOSC value of 76.08 μmol

TE/g bran, while the Shirley wheat bran contained the lowest HOSC value of 48.06 μmol TE/g bran. The present results were almost 2-fold greater than those reported for soft winter wheat flour (Lv et al., 2012). No significant difference with HOSC was found among the ten soft winter wheat cultivars. HOSC value marginally correlated with TPC (r = 0.612, P < 0.001). Whent et al. (2009) also found similar weak correlation between HOSC and TPC in Maryland-grown soybeans. No correlations between HOSC and other radical scavenging capacity assays namely ABTS, RDSC, and ORAC were observed in this study.

2.4.3.4. $ABTS^{\bullet+}$ Scavenging Capacity

Table 2.3. Antioxidant activities of bran samples of 10 Maryland-grown soft winter wheat cultivars [a]

	RDSC	ORAC	HOSC	ABTS
SS520	2.70a ± 0.38	56.71ab ± 6.50	58.18ab ± 8.14	11.90a ± 2.37
SSMPV57	2.56a ± 0.43	56.01ab ± 2.70	63.27ab ± 9.93	12.45a ± 1.45
SS5205	2.49a ± 0.52	61.31b ± 6.34	74.93b ± 14.88	11.76a ± 3.09
USG3555	2.93a ± 0.40	61.50b ± 10.54	75.99b ± 10.01	14.06a ± 2.14
USG3665	3.20a ± 0.13	58.17b ± 6.25	76.08b ± 12.02	14.85a ± 0.61
USG3315	2.77a ± 0.52	48.87ab ± 2.53	62.53ab ± 3.99	12.55a ± 1.55
Branson	2.60a ± 0.56	52.35ab ± 3.22	70.18ab ± 5.88	12.75a ± 1.33
Shirley	2.37a ± 0.40	39.91a ± 7.16	48.06a ± 10.44	11.71a ± 1.38
Jamestown	2.78a ± 0.50	47.75ab ± 11.61	61.73ab ± 14.83	13.14a ± 1.30
Chesapeake	2.18a ± 0.35	47.99ab ± 10.01	58.39ab ± 12.06	12.18a ± 1.15

[a] SS520, SSMPV57, SS5205, USG3555, USG3665, USG3315, Branson, Shirley, Jamestown and Chesapeake are ten soft winter wheat cultivars. RDSC, relative $DPPH^{\bullet}$ radical scavenging capacity; ORAC, oxygen radical absorbance capacity; HOSC, hydroxyl radical scavenging capacity; ABTS, $ABTS^{\bullet+}$ scavenging capacity; TE, Trolox equivalents. Data are expressed as the mean ± SD (n = 3). Unit, μmol TE/g. Means marked with the same letter are not significantly different (P > 0.05).

The $ABTS^{\bullet+}$ scavenging capacity ranged from 11.71 to 14.85 μmol TE/g bran for ten soft winter wheat brans (**Table 2.3**). The present data were comparatively lower than (17.99-18.85 μmol TE/g) bran samples of the two hard wheat cultivars grown in two Colorado locations (Zhou, Yin, & Yu, 2005). However, $ABTS^{\bullet+}$ scavenging capacity was around 5-fold greater than (2.01-2.48 μmol TE/g) soft winter wheat flour (Lv et al., 2012). Just like other scavenging activity reported in the present study, no significant difference ($P < 0.05$) in $ABTS^{\bullet+}$ scavenging capacity among the ten soft winter wheat cultivars at was observed. In addition, the Pearson correlation test showed that $ABTS^{\bullet+}$ scavenging capacity was not correlated with any other antioxidant assays namely, DPPH, HOSC and RDSC or phytochemicals quantified in the present study. This was consistent to the previous reported observation by Moore, Liu, Zhou, & Yu (2006a) where $ABTS^{\bullet+}$ scavenging capacity was not correlated with ORAC in bran samples of the twenty hard winter wheat cultivars grown in two Colorado locations.

2.4.4. Cell-Based Antioxidant Capacity against Hep G2/C3A cells

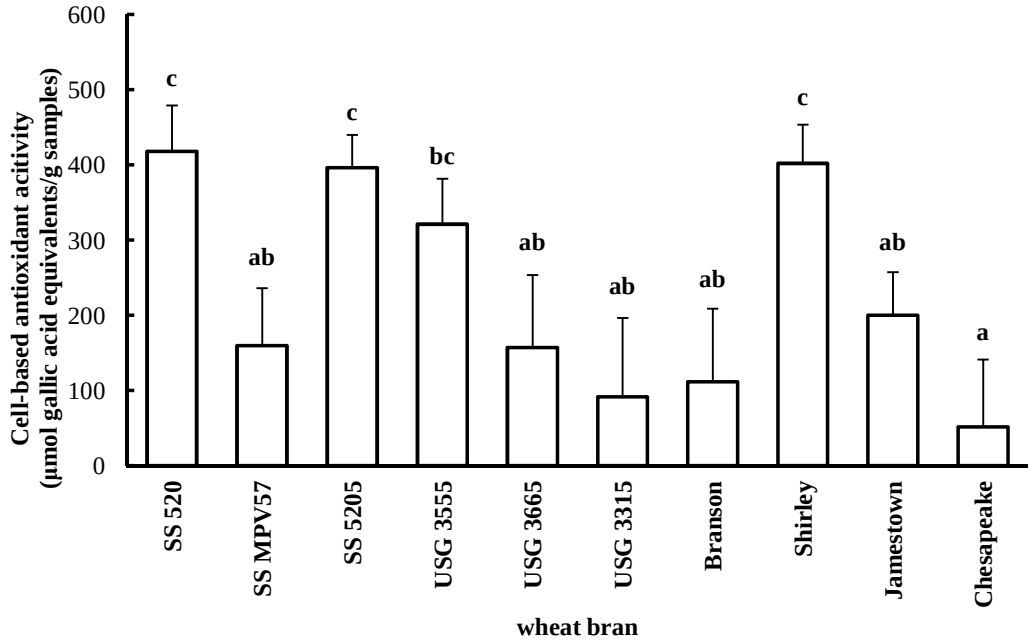

Figure.2.1. Cell-based antioxidant capacity of wheat bran extracts in Hep G2/C3A cells.
SS520, SSMPV57, SS5205, USG3555, USG3665, USG3315, Branson, Shirley, Jamestown and Chesapeake are ten soft winter wheat cultivars. Cell-based antioxidant activity of selected bran samples in Hep G2/C3A cells are expressed as μmol gallic acid equivalents/g (mean ± SEM, n = 6). Bars with the same letters are not significantly different ($P > 0.05$).

In this study, no cytotoxicity was found at 50 mg bran equivalents (BE)/mL of all wheat bran extracts. The SS520 wheat bran had the highest CAA value of 418 μmol of GAE/g bran, while Chesapeake wheat bran exhibited the lowest value of 51.3 μmol of GAE/g bran, respectively (**Figure 2.1**). Significant differences of cell-based antioxidant activities between different wheat bran cultivars were observed. In addition, cellular antioxidant activities of wheat brans showed no correlations with any other radical scavenging capacities.

2.4.5. Antiproliferative Effects of Different Wheat Extracts in HT-29 Cells and Caco-2 Cells

Colon cancer is the third cause of death among men and second cause of death in women (Jemal et al., 2011). A common way that could be used to fight against cancer involves prevention through dietary interventions (Brown et al., 2001). Ferulic acid, the predominant phenolic acid found in wheat grain, has shown antiproliferative effect on HT-29 colon cancer cells (Ferguson, Zhu, & Harris, 2005). All four wheat bran extracts did not significantly inhibit HT-29 cell line at 10.0 mg BE/mL treatment concentration. However, the Jamestown wheat bran exhibited the highest inhibition of HT-29 cancer cells by 28.58% at 50 mg bran equivalents (BE)/mL compared to the vehicle control. At the same concentration, the USG3555, USG3665, and Chesapeake wheat bran samples did not significantly show inhibition of HT-29 cells as compared to Jamestown wheat bran (**Figure 2.2**). This was in agreement with our recent study that soft winter wheat flour could reduce the growth of HT-29 colon cancer cells at a concentration of 50 mg flour equivalents (FE)/mL after 48 hours treatment (Lv et al., 2012). In addition, the extracts of selected whole wheat flour also possessed antiproliferative activity against HT-29 human colorectal carcinoma cells in vitro (Whent et al., 2012).The antiproliferation activities against HT-29 cells was positively correlated with ABTS•+ (r = 0.668, P < 0.05) and ORAC (r = 0.623, P < 0.05), but showed no correlation with any other antioxidant properties or individual phytochemical component.

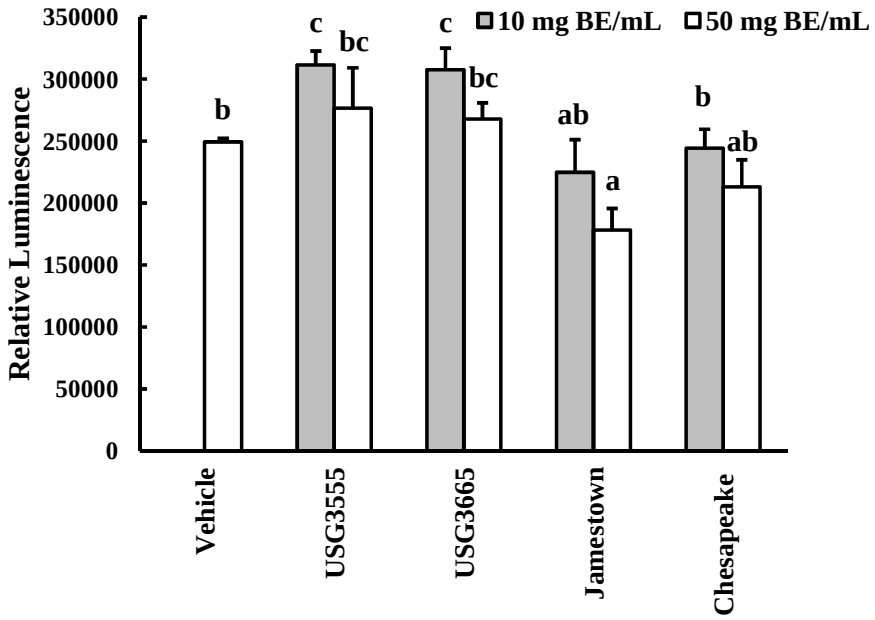

Figure 2.2. Antiproliferative activities of wheat bran in HT-29 human colon cancer cell growth.
USG3555, USG3665, Jamestown and Chesapeake are four selected soft winter wheat cultivars. HT-29 cells (2.5×10^4/mL) was incubated overnight prior to treatments. The final DMSO concentration was 1% (v/v) in the treatment and vehicle. For the lower level treatment, the initial concentration for samples was 10 mg wheat bran equivalents (BE)/mL, whereas the high concentration was 50 mg BE/mL of wheat bran. Data are expressed as the mean of three replicates ± SD (n = 3). Column marked with the same letters are not significantly different ($P > 0.05$).

Caco-2 cells were treated with 10.0 and 50.0 mg BE/mL concentrations of various wheat bran extracts. At 10.0 mg BE/mL concentration, the Jamestown wheat bran inhibited 31% of cells compared to the vehicle control. The USG 3555, USG 3665 and Chesapeake wheat bran did not significantly inhibit Caco-2 cell line at 10.0 mg BE/mL treatment concentration. When treated at 50 mg BE/mL concentration, the Jamestown wheat bran demonstrated the highest inhibition potential, 78% cells were inhibited. In addition, USG3555, USG3665 and Chesapeake wheat bran samples did not significantly inhibit cells as compared to Jamestown wheat bran (**Figure 2.3**). The Pearson correlation test showed that antiproliferation activities of wheat bran samples

against Caco-2 cells was only correlated with $ABTS^{\bullet+}$ ($r = 0.669$, $P < 0.05$) under the experimental conditions.

In general, wheat bran extracts of Jamestown and Chesapeake inhibited colon cancer to some degree. The Jamestown wheat bran significantly inhibited HT-29 and Caco-2 cell lines. Differences in HT-29 and Caco-2 cell lines may be due to efficiency of metabolic uptake within the cell. Mechanisms behind antiproliferative activity within the cell are still difficult to interpret. However, possible reasons include wheat bran samples interfering with proliferative signal transduction within protein kinases of the cell. The previous study showed no evidence of a beneficial or harmful effect for β-carotene in colon cancer in older male smokers, but does provide suggestive evidence that α-tocopherol supplementation may have had a modest prevention effect (Albanes et al., 2000). The results of Pearson correlation test showed that α-tocopherol and β-carotene was not correlated with inhibition of HT-29 and Caco-2 colon cancer cells, respectively (data not included), which partially agreed with the observation of Albanes et al. (2000).

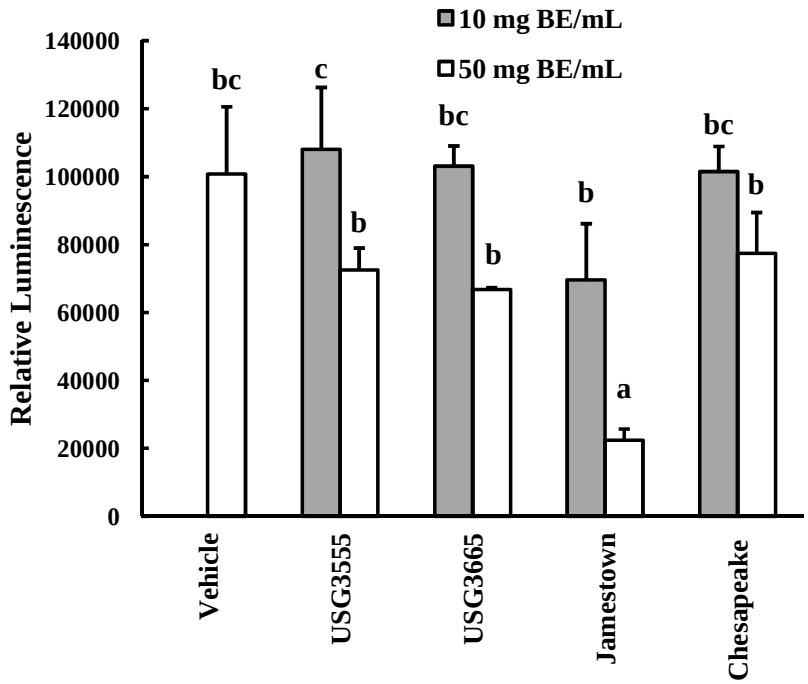

Figure 2.3. Antiproliferative activities of wheat bran in Caco-2 human colon cancer cells.
USG3555, USG3665, Jamestown and Chesapeake are four selected soft winter wheat cultivars. Caco-2 cells (2.5×10^4/mL) was incubated overnight prior to treatments. The final DMSO concentration was 1% (v/v) in the treatment and vehicle. For the lower level treatment, the initial concentration for samples was 10 mg wheat bran equivalents (BE)/mL, whereas the high concentration was 50 mg BE/mL of wheat bran. Data are expressed as the mean of three replicates ± SD (n = 3). Column marked with the same letters are not significantly different ($P > 0.05$).

2.5 Conclusion

In summary, bran samples of all ten Maryland-grown soft winter wheat cultivars contained significant levels of phytochemicals namely, ferulic acid, α- and δ-tocopherols, lutein, zeaxanthin, and β-carotene. All bran extracts showed significant radical scavenging activity. Both phytochemicals content and radical scavenging activity were higher for bran samples as compared to the soft winter wheat flour samples. Insignificant correlations between different radical scavenging activities with identified phytochemical content were observed. The Jamestown wheat bran had the strongest antiproliferation activity against both HT-29 and Caco-2 cancer cells.

Results from this study indicate that additional research is needed for comparing radical scavenging activities by different assays and its relation to health and nutrition.

Chapter 3: Influences of genotype, environment, and their interaction on the phytochemical compositions and antioxidant properties of soft winter wheat bran

3.1 Abstract

The nutritional quality of wheat (*Triticum aestivum* L.) is dependent on both genetic and environmental factors. The influences of genotype (G), growing environment (E), and their interaction (G × E) on the phytochemical compositions and antioxidant properties of ten soft winter wheat bran cultivars grown in four locations were investigated. In general, larger variability for selected health beneficial components and antioxidant properties of soft winter wheat bran were attributed more by E than among G and G ×E ($P < 0.001$). E had a strong impact on α-tocopherol, δ-tocopherol, total tocopherols, total phenolic content (TPC), total soluble ferulic acid, and $ABTS^{\bullet+}$ cation and $DPPH^{\bullet}$ radical scavenging capacities ($P < 0.001$). Our study also showed that each soft wheat bran component or antioxidant property may respond to individual environmental factors differently. For the first time, these results showed that E, G, and G × E could affect differently the levels of selected health components and antioxidant properties of soft winter wheat bran.

3.2 Introduction

Wheat (*Triticum aestivum* L.) is mainly categorized into hard and soft classes according to its agronomic and end-use attributes, and its consumption is increasing world-wide (USDA, 2013a). Soft wheat, especially soft red wheat, accounting for 15-20 percent of total production in US, is grown primarily in States along with Mississippi river, and is the most important wheat with highest consumption in

eastern states (USDA, 2013b). Growing evidence indicates that wheat and wheat-based food commodities contain health-beneficial components including natural antioxidants, such as ferulic acid (Liyana-Pathirana & Shahidi, 2006b; Mateo Anson et al., 2008), lutein (Adom, Sorrells, & Liu, 2005) and α-tocopherol (Zhou, Su, & Yu, 2004a), which are the predominant phenolic acid, carotenoid and tocopherol present in wheat grain, respectively, as well as potential cholesterol-lowering components (Cheng et al., 2008). Antioxidant intake has been linked in epidemiological studies to a reduced risk of chronic diseases including cardiovascular diseases and cancer (Wilcox, Ash, & Catignani, 2004).

The phytochemical compositions and antioxidant properties of wheat as recognized by the wheat breeder is a product of both genetic and environmental influences (William et al., 2008). Mpofu, Sapirstein, & Beta (2006) reported that total phenolic content (TPC), $DPPH^{\bullet}$ scavenging capacity, and vanillic, syringic and ferulic acids contents of hard spring wheat were more altered by growing environment (E), while genotype (G) contributed more to caffeic and *p*-coumaric acid contents. The genotype × growing environment (G × E) interaction contributed up to 6.71% influence to the tested health component under the experimental conditions. In addition, our previous study also showed that TPC, levels of individual phenolic acids and scavenging capacities against $ABTS^{\bullet+}$ and $O_2^{\bullet-}$ of hard wheat bran were primarily controlled by environment, with E being generally a much greater source of variation than G and G × E (Moore, Liu, Zhou, & Yu, 2006a). Different fracture patterns in soft and hard wheat may result in different levels of phytochemical compositions. Soft wheat that has less damage during milling because of its smaller particle size may also have varied levels of health-beneficial components compared with hard wheat. These

suggested that the phytochemical compositions of soft wheat might be altered differently by G, E and their interaction as well.

Our recent study investigated the effect of G, E and G × E on the antioxidant properties and chemical compositions of soft winter wheat flour, and found that E had the largest effect on antioxidant activity against oxygen, hydroxyl and $ABTS^{\bullet+}$ radicals as well as total carotenoids contents, while G × E interaction had a larger effect on the level of total tocopherols (71.6%) (Lv et al., 2013). However, natural antioxidants are condensed mostly in the wheat bran fraction (Moore et al., 2005). While there is previous literature describing the composition of both hard and soft wheat, however, the effect of genotype, growing environment and their interaction on health properties in soft wheat bran has not been reported. As a continuation of our recent study on soft winter wheat (Lv et al., 2012; Lv et al., 2013), this study was the first time that conducted to determine whether and how G, E, and G × E may alter phenolic acids, carotenoids, tocopherols, and antioxidant properties of soft winter wheat bran. The effects of environmental factors including precipitation and temperature stress on the antioxidant properties and phytochemical contents of soft winter wheat bran were also investigated. The understanding of the G, E and G× E effects on wheat health properties is essential, and can be used for improving the breeding efforts to produce soft wheat cultivars rich in selected health components to meet market needs.

3.3 Materials and Methods

3.3.1. Materials

Ten soft red winter wheat (*Triticum aestivum* L.) cultivars, SS520, SSMPV57, SS5205, USG3555, USG3665, USG3315, Branson, Shirley, Jamestown, and

Chesapeake were included in the study. These genotypes, that represent a sample of elite commercial cultivars currently grown in the mid-Atlantic, were grown in the field during the 2011 growing season at four testing locations (Clarksville, Keedysville, Poplar Hill (Quantico) and Wye (Queenstown)) in Maryland, in yield trial plots 4 m long by 1 m wide at a density of approximately 350,000 plants/ha. Plots were planted following a crop of corn in October 2010. Plots were fertilized with an autumn application of 16 kg/ha of nitrogen, 40 kg/ha of phosphorus, and 80 kg/ha of potassium. Additionally, 30–80 kg/ha of nitrogen was applied in March or April 2011 (depending on location). Grain from the field plots was mechanically harvested, threshed, and cleaned of debris prior to laboratory testing.

3.3.2. Chemicals and Reagents

Disodium ethylenediaminetetraacetate (EDTA), 2, 2'-bipyridyl, 2, 2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$), fluorescein (FL), lauryl sulfate sodium salt, sodium hydroxide, ethyl ether, ethyl acetate, 6-hydroxy-2, 5, 7, 8- tetramethylchroman-2-carboxylic acid (Trolox), tocopherols (α-, δ-, and γ-), ascorbic acid, and β-carotene were purchased from Sigma–Aldrich (St. Louis, MO, USA). Iron (III) chloride, $ABTS^{TM}$ chromophore, diammonium salt and thirty percent ACS-grade hydrogen peroxide were obtained from Fisher Scientific (Fair Lawn, NJ, USA). 2, 2'-azinobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals (Richmond, VA, USA). Ultrapure water was used for all experiments, which was prepared by an ELGA Purelab ultra Genetic polishing system with < 5 ppb TOC and resistivity of 18.2 mΩ (Lowell, MA, USA). All other chemicals and solvents were of the highest commercial grade and used without further purification.

3.3.3. Preparation and Extraction of Soft Wheat Bran

Each wheat sample was ground to a particle size of 40-mesh using a handheld coffee grinder and separated into flour and bran fraction. The bran yield was about 17.0-22.2 %. The milled brans were kept in a -20 ^{o}C freezer in airtight containers until analysis. Half gram of ground wheat bran sample was extracted with 5 mL of 50% acetone for 24 hours under nitrogen at ambient temperature. The acetone extracts were used for estimating total phenolic content (TPC), hydroxyl radical scavenging capacity (HOSC), capacity relative DPPH$^{\bullet}$ scavenging capacity (RDSC), oxygen radical absorbing capacity (ORAC), and ABTS$^{\bullet+}$ scavenging capacity. The extracts were stored under nitrogen in dark at ambient temperature until further analysis.

3.3.4. Total Phenolic Content (TPC)

The TPC of wheat bran was determined according to a laboratory procedure described previously (Yu et al., 2002). In general, the final reaction mixture contained 50 µL wheat bran extract, 250 µl of the Folin–Ciocalteu reagent, 750 µL of 20% sodium carbonate, and 3 mL ultrapure water. Gallic acid was used as the standard. After 2 hours of reaction at ambient temperature in dark, absorbance was read at 765 nm. The results were expressed as mg of gallic acid equivalent (GAE)/g of wheat bran on a dry weight basis.

3.3.5. Total soluble Ferulic Acid Content of Soft Wheat Bran

Each wheat bran sample was analyzed for its total soluble ferulic acid including soluble free and conjugated ferulic acids according to the laboratory method described by Moore et al. (2005). Ground wheat bran were extracted with

acetone/methanol/water (7:7:6, v/v/v) to obtain the soluble supernatant. Soluble free and conjugated ferulic acids in the supernatants were separated on the basis of their solubility under acidic condition (pH = 2), and the conjugated ferulic acid was released by acidic hydrolysis (Moore et al., 2005). Free ferulic acid was extracted with ethyl ether and ethyl acetate (1:1, v/v). After evaporating the organic phase under nitrogen, each extract was re-dissolved in methanol and filtered through a 0.45 µm membrane filter, and subjected to HPLC analysis. The mobile phase A consisted of acetic acid/H_2O (2:98, v/v) and mobile phase B consisted of acetic acid/acetonitrile /H_2O (2:30:68, v/v/v). Elution was programmed from 10 to 100% B in 42 min with a flow rate of 1.0 mL/min. Injection volume was 10 µl. Quantification was based on the area under the peak of external standards. The results were expressed as µg/g of wheat bran on a dry weight basis.

3.3.6. Carotenoid Composition

Two hundred mg of ground wheat bran was extracted with 10 mL of methanol/tetrahydrofuran (1:1, v/v) for 15 hours at ambient temperature. The resulting extraction mixture was centrifuged at 2,000 rpm. The solvent was removed from the supernatants under N_2. The residues were re-dissolved in 2 mL of methanol/acetonitrile/iso-propanol (54:44:2, v/v/v), filtered through a 0.45 µm membrane filter, and analyzed for carotenoids using a HPLC. HPLC separation was accomplished using a Shimadzu LC-20AD with an autosampler, a Phenomenex C18 column (4.6 mm i.d. × 250 mm, 5 µm particle size) at 25 ^oC and a UV-VIS detector at 450 nm, according to a previously described laboratory protocol (Moore et al., 2005). The carotenoids were eluted using a mobile phase of water as solvent A and methanol/acetonitrile/iso-propanol (54:44:2, v/v/v) as solvent B. The gradient

procedure was as follows: 1) the gradient was linear from 95% to 99% of solvent B in the first 10 min, 2) 99% of solvent B for 10 min, and 3) the gradient was linear from 99% to 95% of solvent B for the last 5 min. The flow rate was 1.0 mL/min and 20 µL of each standard or sample was injected. A standard curve was developed from the known standards, and peak area was used for quantification. The results were expressed as µg/g of wheat bran on a dry weight basis.

3.3.7. Tocopherol Content

HPLC analysis was accomplished using a Shimadzu LC-20AD with an autosampler and an UV-VIS detector, and a Phenomenex C18 column (4.6 mm i.d. × 250 mm, 5 µm particle size) at 25 °C according to a previously described protocol (Zhou, Yin, & Yu, 2005). The tocopherols were separated using an isocratic elution with a mobile phase 1% solvent A (water) and 99% solvent B (acetonitrile), with a flow rate of 1.5 mL/min. Injection volume was 20 µL. A standard curve was developed from the known standards (α-, δ-, and γ-tocopherol), and peak areas were used for quantification. The results were expressed as µg/g of bran on a dry weight basis.

3.3.8. Hydroxyl Radical (HO•) Scavenging Capacity (HOSC)

The HOSC assay was performed according to a previously reported laboratory procedure using a Victor^3 multi-label plate reader (PerkinElmer, Turku, Finland) (Moore, Yin, & Yu, 2006b). In brief, the reaction mixture consisted of 170 µL of 9.28 × 10^{-8} M fluorescein (FL), 30 µL bran sample, blank, or Trolox standard, 40 µL of freshly prepared 0.1990 M H_2O_2 and 60 µL of 3.43 mM $FeCl_3$. The fluorescence was

recorded every 4 min for 4 hours. The HOSC was calculated from the area under the curve and the results were expressed as µmol Trolox equivalent (TE)/g of wheat bran on a dry weight basis.

3.3.9. Relative DPPH• Radical Scavenging Capacity (RDSC)

The RDSC values were determined according to a previously described laboratory procedure (Cheng, Moore, & Yu, 2006). Briefly, the final reaction mixture contained 100 µL bran extract, Trolox standard or solvent (positive and negative controls), and 100 µL of 0.2 mM working DPPH• solution. The absorbance was measured at 515 nm every minute for 40 min, using a Victor^3 multi-label plate reader (PerkinElmer, Turku, Finland). The RDSC was calculated from the area under the curve and the results were expressed as µmol TE/g of wheat bran on a dry weight basis.

3.3.10. Oxygen Radical Absorbance Capacity (ORAC)

The ORAC values were determined following a previously reported laboratory protocol using a Victor^3 multi-label plate reader (Perkin-Elmer, Turku, Finland) (Moore et al., 2005), with FL as the fluorescent probe. Briefly, the initial reaction mixture containing 225 µL of 8.16×10^{-8} M FL, and 30 µL bran sample, blank or standard, were preheated in the plate reader at 37 °C for 20 min. Then 25 µL of 0.36 M AAPH was added to each well and the fluorescent of the mixture was recorded every 2 min over 2 hours at 37 °C. Trolox was used as a standard. The results were expressed as µmol TE/g of wheat bran on a dry weight basis.

3.3.11. $ABTS^{\bullet+}$ Scavenging Capacity

The scavenging ability against $ABTS^{\bullet+}$ of wheat bran extract was measured using a previously reported protocol (Zhou, Laux, & Yu, 2004b). $ABTS^{\bullet+}$ radicals were generated by oxidizing a 5 mM aqueous solution ABTS with manganese dioxide under ambient temperature for 30 min. The final reaction mixture contained 1.0 mL $ABTS^{\bullet+}$ solution with an absorbance of 0.700 ± 0.005 at 734 nm and 80 µl of 50% acetone for the control or 80 µL of the wheat bran sample or standard solution. The absorbance was read at 734 nm after 90 s of reaction. Trolox was used as a standard. Results were calculated using a standard curve and expressed as µmol TE/g of wheat bran on a dry weight basis.

3.3.12. Weather Data

The precipitation and daily temperature highs, lows and averages at each location during growing season were provided by Dr. Jose Costa. Grain filling time was calculated from heading date until harvest date for each wheat genotype and location. Average high, average low, overall average temperature, and precipitation level were recorded for each location during the grain filling period and are presented in **Table 3.1.** The Wye Research Center is a coastal location on the Chesapeake Bay in Maryland. The Poplar Hill and Keedysville location is 60 and 110 miles northwest of the Wye Research Center, respectively. The Clarksville is 50 miles west of the Wye Research Center.

Table 3.1. Environmental conditions during wheat grain filling time [a]

	Abs. high (°F)	Abs. low (°F)	Avg. high (°F)	Avg. low (°F)	Overall Avg. (°F)	Precip (inches)
SS520/PH	97.00	40.01	80.16	57.90	69.03	0.04
SS520/W	92.32	45.93	79.08	60.84	69.96	0.10
SS520/CV	95.90	48.90	81.47	60.59	71.03	0.08
SS520/KV	94.00	52.00	80.00	61.68	70.84	0.11
SSMPV57/PH	97.00	40.01	80.64	58.43	69.54	0.04
SSMPV57/W	92.32	45.93	78.65	60.44	69.55	0.10
SSMPV57/CV	95.90	48.90	83.24	60.83	72.04	0.05
SSMPV57/KV	94.00	52.00	81.95	62.56	72.26	0.05
SS5205/PH	97.00	40.01	80.51	58.31	69.41	0.04
SS5205/W	92.32	45.93	79.03	60.73	69.88	0.10
SS5205/CV	95.90	48.90	82.30	60.73	71.52	0.08
SS5205/KV	94.00	52.00	80.11	61.89	71.00	0.11
USG3555/PH	97.00	40.01	80.62	58.44	69.53	0.04
USG3555/W	92.32	45.93	79.03	60.73	69.88	0.10
USG3555/CV	95.90	48.90	81.86	60.67	71.27	0.08
USG3555/KV	94.00	52.00	81.41	62.20	71.81	0.08
USG3665/PH	97.00	40.01	80.62	58.44	69.53	0.04
USG3665/W	92.32	45.93	79.03	60.73	69.88	0.10
USG3665/CV	95.90	48.90	82.30	60.73	71.52	0.08
USG3665/KV	94.00	52.00	81.65	62.35	72.00	0.06
USG3315/PH	97.00	40.01	80.64	58.43	69.54	0.04
USG3315/W	92.32	45.93	79.03	60.73	69.88	0.10
USG3315/CV	95.90	48.90	82.43	60.76	71.60	0.08
USG3315/KV	94.00	52.00	81.65	62.35	72.00	0.06
Branson/PH	97.00	40.01	80.16	57.90	69.03	0.04
Branson/W	92.32	45.93	79.09	60.84	69.96	0.10
Branson/CV	95.90	48.90	81.32	60.34	70.83	0.08
Branson/KV	94.00	52.00	80.42	61.93	71.18	0.11
Shirley/PH	97.00	40.01	80.51	58.31	69.41	0.04
Shirley/W	92.32	45.93	79.03	60.73	69.88	0.10
Shirley/CV	95.90	48.90	82.30	60.73	71.52	0.08
Shirley/KV	94.00	52.00	81.65	62.35	72.00	0.06
Jamestown/PH	97.00	40.01	80.51	58.31	69.41	0.04
Jamestown/W	92.32	45.93	79.08	60.84	69.96	0.10
Jamestown/CV	95.90	48.90	81.32	60.34	70.83	0.08
Jamestown/KV	94.00	52.00	80.42	61.93	71.18	0.11
Chesapeake/PH	97.00	40.01	80.51	58.31	69.41	0.04
Chesapeake/W	92.32	45.93	79.12	60.87	70.00	0.10
Chesapeake/CV	95.90	48.90	81.47	60.59	71.03	0.08
Chesapeake/KV	94.00	52.00	80.77	62.00	71.39	0.11

[a] Temperatures reported for each location and genotype represent absolute high and low, average high and low, and overall average in °F during 2011 wheat grain filling time.

3.3.13. Statistical Analysis

The data was reported as Mean ± SD (n = 3). Differences between means were determined by analysis of variance (ANOVA) with Tukey's HSD post hoc test ($P < 0.05$), using SPSS (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL, USA). Factorial design ANOVA was performed using a general linear model (GLM) to determine the contribution of genotype and environment to variance, using genotype and environment as fixed effects. Correlation analyses were performed using a two-tailed Pearson's correlation test. Statistical significance was declared at $P < 0.05$.

3.4 Results and Discussion

3.4.1. Phytochemical Compositions of Bran Samples of 10 Soft Winter Wheat Cultivars Grown at Four Locations

USG3555 wheat bran grown in Clarksville location had the greatest total phenolic content (TPC) of 2.81 mg GAE/g bran, whereas the lowest TPC of 1.82 mg GAE/g bran was observed in the SS520 wheat bran from Wye location (**Figure 3.1**). This range was comparable to 2.2-2.9 mg GAE/g for seven wheat bran samples from four different countries (Zhou, Su, & Yu, 2004a) and 2.7-3.5 mg GAE/g for bran samples of the twenty hard winter wheat cultivars grown in the two Colorado locations (Moore, Liu, Zhou, & Yu, 2006a).

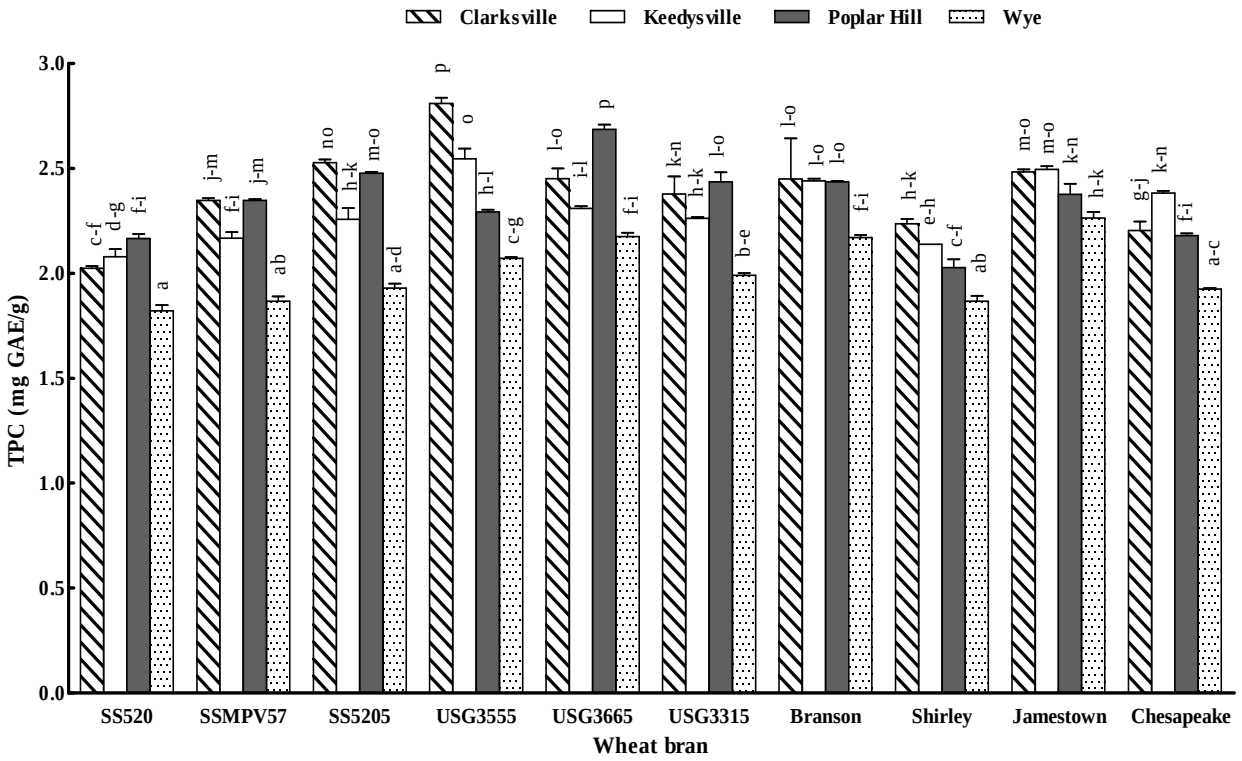

Figure 3.1. Total phenolic content (TPC) of bran samples of the ten soft winter wheat cultivars grown in four locations.
SS520, SSMPV57, SS5205, USG3555, USG3665, USG3315, Branson, Shirley, Jamestown, and Chesapeake are ten soft winter wheat cultivars. Clarksville, Keedysville, Poplar Hill, and Wye represent the growing locations. TPC stands for total phenolic content. Data are expressed as micrograms gallic acid equivalents (GAE)/g of wheat bran. Vertical bars represent the mean of three replicate plots ± SD (n = 3). Values marked by the different letters are statistically different ($P < 0.05$).

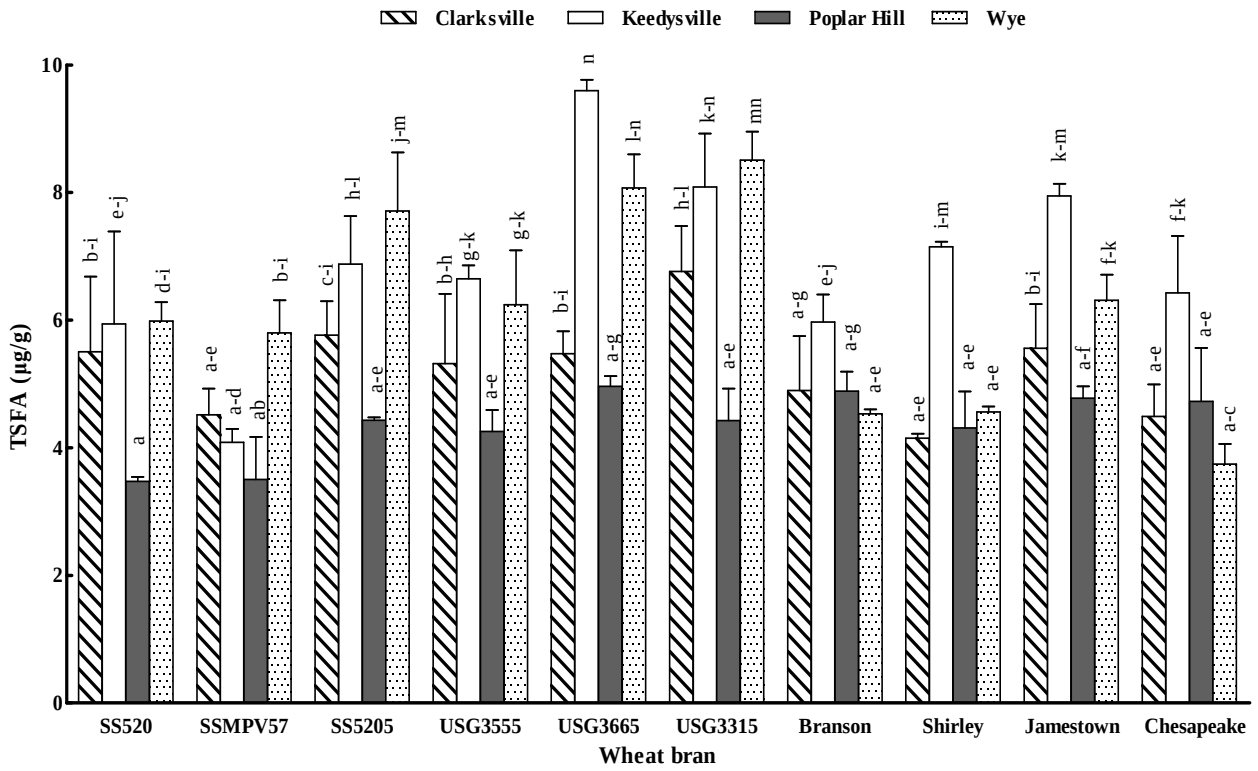

Figure 3.2. Total soluble ferulic acid (TSFA) content of bran samples of the ten soft winter wheat cultivars grown in four locations.

SS520, SSMPV57, SS5205, USG3555, USG3665, USG3315, Branson, Shirley, Jamestown, and Chesapeake are ten soft winter wheat cultivars. Clarksville, Keedysville, Poplar Hill, and Wye are the growing locations. TSFA stands for total soluble ferulic acid. Data are expressed as micrograms per gram of wheat bran. Vertical bars represent the mean of three replicate plots ± SD (n = 3). Values marked by the different letters are statistically different ($P < 0.05$).

As shown in **Figure 3.2**, USG3665 wheat bran from Keedysville location had the greatest total soluble ferulic acid content of 9.60 µg/g bran, whereas the lowest total soluble ferulic acid content of 3.48 µg/g bran was observed in the SS520 wheat bran from Poplar Hill location. Interestingly, compared to their counterparts grown in the other three locations, bran samples of the ten wheat cultivars from Poplar Hill location had lower or the same content of total soluble ferulic acid, indicating that growing environment might affect the total soluble ferulic acid content in the soft wheat brans.

Lutein, zeaxanthin and *β*-carotene were detected in all wheat bran samples **(Table 3.2).** Lutein was the primary carotenoid in all forty soft wheat bran samples with a concentration ranging from 0.47 to 1.65 µg/g of bran. Zeaxanthin concentration varied from 0.10 to 0.41 µg/g bran, while the level of *β*-carotene ranged from 0.02 to 0.28 µg/g bran. The lutein and zeaxanthin concentrations detected in this study were comparable to 1.64-1.92 and 0.19-0.26 µg/g, respectively, for the soft wheat bran samples reported by Adom, Sorrells, & Liu (2005) and 0.82-1.14 µg/g for lutein and 0.20-0.39 µg/g for zeaxanthin in Maryland-grown soft wheat grains (Moore et al., 2005). In addition, except for Shirley cultivar, bran samples of other nine wheat cultivars from Wye location had higher or same levels of lutein and total carotenoids, ranging from 1.34-1.75 µg/g and 3.18-4.02 µmol/kg bran, respectively, compared to their counterparts grown in the other three locations, suggesting a potential effect of growing conditions on wheat carotenoid composition.

Table 3.2. Carotenoid composition of bran samples of the ten soft winter wheat cultivars grown at four locations [a]

	Lutein (µg/g)	Zeaxanthin (µg/g)	β-Carotene (µg/g)	Total Caros (µmol/kg)
SS520/PH	1.15 ± 0.01 g-j	0.29 ± 0.01 i-p	0.03 ± 0.00 a-c	2.57 ± 0.01 f-i
SS520/W	1.44 ± 0.01 q	0.32 ± 0.00 n-q	0.25 ± 0.01 rs	3.56 ± 0.02 n
SS520/CV	1.38 ± 0.03 pq	0.31 ± 0.01 l-q	0.05 ± 0.00 de	3.08 ± 0.06 mn
SS520/KV	1.07 ± 0.01 e-h	0.25 ± 0.01 f-k	0.15 ± 0.00 lm	2.61 ± 0.02 ef
SSMPV57/PH	1.19 ± 0.02 i-l	0.30 ± 0.02 k-p	0.04 ± 0.00 bc	2.68 ± 0.05 h-j
SSMPV57/W	1.63 ± 0.01 rs	0.38 ± 0.01 st	0.19 ± 0.01 o	3.90 ± 0.03 p
SSMPV57/CV	1.33 ± 0.03 n-p	0.29 ± 0.01 i-o	0.04 ± 0.00 cd	2.92 ± 0.05 k-m
SSMPV57/KV	1.23 ± 0.03 j-m	0.24 ± 0.02 e-j	0.11 ± 0.01 j	2.80 ± 0.01 h-j
SS5205/PH	1.25 ± 0.04 k-n	0.32 ± 0.01 o-q	0.09 ± 0.00 i	2.94 ± 0.07 j-l
SS5205/W	1.37 ± 0.02 o-q	0.32 ± 0.02 m-q	0.22 ± 0.01 p	3.37 ± 0.02 mn
SS5205/CV	0.74 ± 0.01 b	0.17 ± 0.01 bc	0.13 ± 0.00 k	1.85 ± 0.02 bc
SS5205/KV	1.19 ± 0.02 i-l	0.26 ± 0.03 f-k	0.11 ± 0.00 j	2.75 ± 0.06 g-i
USG3555/PH	0.93 ± 0.03 d	0.22 ± 0.02 e-f	0.06 ± 0.00 e-g	2.14 ± 0.02 d
USG3555/W	1.61 ± 0.09 rs	0.41 ± 0.04 t	0.07 ± 0.00 gh	3.69 ± 0.23 p
USG3555/CV	1.36 ± 0.01 n-p	0.30 ± 0.00 k-p	0.08 ± 0.00 hi	3.07 ± 0.02 l-n
USG3555/KV	1.23 ± 0.02 j-m	0.28 ± 0.01 h-n	0.25 ± 0.00 s	3.12 ± 0.05 h-k
USG3665/PH	0.69 ± 0.00 b	0.14 ± 0.00 b	0.02 ± 0.00 a	1.50 ± 0.01 b
USG3665/W	1.34 ± 0.03 n-p	0.33 ± 0.01 p-r	0.13 ± 0.00 k	3.18 ± 0.05 l-n
USG3665/CV	1.05 ± 0.01 e-g	0.23 ± 0.02 e-h	0.05 ± 0.00 de	2.35 ± 0.05 e
USG3665/KV	1.25 ± 0.04 k-n	0.27 ± 0.01 g-m	0.25 ± 0.01 s	3.16 ± 0.10 i-k
USG3315/PH	0.88 ± 0.05 d	0.22 ± 0.01 d-f	0.13 ± 0.00 k	2.18 ± 0.10 d
USG3315/W	1.75 ± 0.04 t	0.38 ± 0.02 st	0.09 ± 0.00 i	3.91 ± 0.10 q
USG3315/CV	1.19 ± 0.02 i-l	0.28 ± 0.02 h-o	0.11 ± 0.00 j	2.78 ± 0.05 h-j
USG3315/KV	1.65 ± 0.10 s	0.38 ± 0.02 st	0.13 ± 0.00 k	3.81 ± 0.14 pq
Branson/PH	1.28 ± 0.03 l-o	0.29 ± 0.01 i-p	0.14 ± 0.00 kl	3.01 ± 0.06 j-l
Branson/W	1.54 ± 0.02 r	0.35 ± 0.01 q-s	0.24 ± 0.00 qr	3.77 ± 0.04 o
Branson/CV	0.77 ± 0.02 b	0.17 ± 0.01 bc	0.07 ± 0.00 fh	1.79 ± 0.06 c
Branson/KV	1.18 ± 0.03 i-l	0.24 ± 0.01 e-h	0.17 ± 0.00 n	2.81 ± 0.03 f-i
Shirley/PH	1.14 ± 0.01 f-j	0.26 ± 0.01 f-k	0.05 ± 0.01 cd	2.54 ± 0.01 f-h
Shirley/W	1.15 ± 0.02 h-k	0.29 ± 0.01 i-p	0.15 ± 0.00 lm	2.82 ± 0.06 g-i
Shirley/CV	1.04 ± 0.05 e-f	0.23 ± 0.01 e-g	0.05 ± 0.00 de	2.33 ± 0.08 e
Shirley/KV	1.59 ± 0.03 rs	0.37 ± 0.01 r-t	0.17 ± 0.01 n	3.76 ± 0.04 op
Jamestown/PH	0.47 ± 0.02 a	0.10 ± 0.00 a	0.03 ± 0.00 ab	1.04 ± 0.04 a
Jamestown/W	1.40 ± 0.03 pq	0.31 ± 0.01 l-q	0.12 ± 0.00 jk	3.23 ± 0.04 m-n
Jamestown/CV	1.10 ± 0.03 e-i	0.24 ± 0.00 e-i	0.08 ± 0.00 hi	2.51 ± 0.05 e-g
Jamestown/KV	0.86 ± 0.03 cd	0.21 ± 0.01 c-e	0.28 ± 0.01 t	2.40 ± 0.04 d
Chesapeake/PH	1.30 ± 0.04 m-p	0.27 ± 0.02 g-l	0.06 ± 0.00 ef	2.89 ± 0.06 j-l
Chesapeake/W	1.66 ± 0.04 n-p	0.38 ± 0.02 st	0.24 ± 0.00 pq	4.02 ± 0.07 pq
Chesapeake/CV	1.02 ± 0.01 e	0.25 ± 0.04 f-k	0.16 ± 0.00 mn	2.54 ± 0.05 e
Chesapeake/KV	0.78 ± 0.02 bc	0.18 ± 0.02 b-d	0.08 ± 0.01 f-h	1.82 ± 0.09 c

[a] Values marked by the different letters are statistically different ($P < 0.05$).

α-Tocopherol concentration ranged from 2.58 to 10.56 µg/g bran in the tested wheat bran samples, which was comparable to 4.10-6.51 µg/g for bran samples of the two hard winter wheat cultivars grown in two Colorado locations (Zhou, Yin, & Yu, 2005). In addition, Shirley wheat bran from Wye location contained the highest α-tocopherol content among all tested wheat bran samples. δ-Tocopherol was presented in all tested wheat bran samples except that of SS520 wheat from Poplar Hill location, ranging from 0.05 to 0.24 µg/g bran. This was comparable to that of 0.16-0.38 µg/g for Colorado hard red winter wheat bran grown in two locations (Zhou, Yin, & Yu, 2005). The content of total tocopherols in wheat bran samples varied from 6.00 to 24.82 µmol/kg bran, comparable to that of 18.7-29.5 µmol/kg bran in the hard red wheat brans. Interestingly, all ten wheat cultivars grown in Poplar Hill location had lower or same concentration of α-, δ- and total tocopherols than their counterparts grown in the other three locations, while bran samples of all ten soft wheat cultivars from Wye location had higher or same levels of α-tocopherol and total tocopherols, compared to their counterparts grown in the other three locations. This indicated that growing environment might contribute major effect on α-tocopherol and total tocopherol levels of soft winter wheat brans **(Table 3.3).** Taking together, these results indicated that both wheat cultivar and growing environment, especially the later factor might have significant effects on the phytochemical compositions of soft winter wheat bran.

Table 3.3. Tocopherol composition of bran samples of the ten soft winter wheat cultivars grown at four locations [a]

	α-Tocopherol (µg/g)	δ-Tocopherol (µg/g)	Total Tocos (µmol/kg)
SS520/PH	2.58 ± 0.04 a	nd	6.00 ± 0.09 a
SS520/W	6.97 ± 0.30 f-j	0.07 ± 0.00 b	16.36 ± 0.70 e-h
SS520/CV	6.78 ± 0.36 f-h	0.22 ± 0.01 jk	16.08 ± 0.85 e-g
SS520/KV	7.78 ± 0.21 i-m	0.12 ± 0.00 q-s	18.61 ± 0.49 h-l
SSMPV57/PH	3.64 ± 0.04 bc	0.09 ± 0.00 c-e	8.68 ± 0.09 b
SSMPV57/W	8.18 ± 0.14 lm	0.17 ± 0.02 lm	19.38 ± 0.34 kl
SSMPV57/CV	6.44 ± 0.07 e-g	0.15 ± 0.01 i-k	15.30 ± 0.16 d-f
SSMPV57/KV	6.92 ± 0.21 f-j	0.23 ± 0.01 pq	16.58 ± 0.47 e-i
SS5205/PH	4.27 ± 0.03 cd	0.09 ± 0.00 b-d	10.11 ± 0.07 bc
SS5205/W	7.44 ± 0.15 g-l	0.11 ± 0.01 no	17.72 ± 0.37 g-k
SS5205/CV	6.55 ± 0.05 e-g	0.14 ± 0.01 f-h	15.50 ± 0.13 d-g
SS5205/KV	7.02 ± 0.19 f-k	0.22 ± 0.01 p-r	16.81 ± 0.44 f-j
USG3555/PH	3.35 ± 0.32 ab	0.10 ± 0.00 de	8.01 ± 0.73 b
USG3555/W	6.00 ± 0.34 ef	0.20 ± 0.01 op	14.40 ± 0.81 de
USG3555/CV	5.66 ± 0.33 e	0.12 ± 0.01 h-k	13.46 ± 0.78 d
USG3555/KV	6.04 ± 0.25 ef	0.22 ± 0.01 mn	14.45 ± 0.60 d-f
USG3665/PH	4.27 ± 0.30 cd	0.10 ± 0.00 de	10.15 ± 0.70 bc
USG3665/W	6.84 ± 0.39 f-i	0.20 ± 0.00 s	16.46 ± 0.91 e-i
USG3665/CV	6.40 ± 0.01 ef	0.14 ± 0.01 kl	14.28 ± 1.60 de
USG3665/KV	6.88 ± 0.31 f-j	0.22 ± 0.00 p-r	16.48 ± 0.70 e-i
USG3315/PH	4.81 ± 0.11 d	0.11 ± 0.00 d-f	11.42 ± 0.28 c
USG3315/W	8.69 ± 0.23 mn	0.24 ± 0.01 mn	20.59 ± 0.54 lm
USG3315/CV	6.40 ± 0.28 ef	0.16 ± 0.00 g-j	15.15 ± 0.65 d-f
USG3315/KV	9.35 ± 0.30 no	0.29 ± 0.00 t	22.38 ± 0.71 mn
Branson/PH	4.61 ± 0.62 d	0.08 ± 0.00 bc	10.87 ± 1.45 c
Branson/W	7.84 ± 0.17 j-m	0.18 ± 0.00 jk	18.53 ± 0.40 h-l
Branson/CV	6.79 ± 0.70 f-h	0.13 ± 0.01 h-j	16.07 ± 1.63 e-g
Branson/KV	7.94 ± 0.23 k-m	0.24 ± 0.00 q-s	18.99 ± 0.53 j-l
Shirley/PH	6.11 ± 0.16 ef	0.09 ± 0.00 c-e	14.41 ± 0.38 de
Shirley/W	10.56 ± 0.32 p	0.14 ± 0.01 g-i	24.82 ± 0.72 o
Shirley/CV	6.76 ± 0.32 f-h	0.13 ± 0.01 h-j	16.01 ± 0.77 e-g
Shirley/KV	6.96 ± 0.31 f-j	0.20 ± 0.00 no	16.60 ± 0.72 e-i
Jamestown/PH	3.50 ± 0.33 bc	0.05 ± 0.00 a	8.26 ± 0.76 b
Jamestown/W	7.74 ± 0.45 h-m	0.13 ± 0.02 m-o	18.40 ± 1.06 h-l
Jamestown/CV	6.48 ± 0.33 e-g	0.14 ± 0.01 h-k	15.36 ± 0.78 d-f
Jamestown/KV	7.86 ± 0.28 j-m	0.20 ± 0.01 no	18.70 ± 0.68 i-l
Chesapeake/PH	4.68 ± 0.14 d	0.11 ± 0.01 e-g	11.13 ± 0.35 c
Chesapeake/W	10.09 ± 0.78 op	0.19 ± 0.00 p-r	23.94 ± 1.81 no
Chesapeake/CV	6.37 ± 0.06 ef	0.14 ± 0.01 f-h	15.08 ± 0.15 d-f
Chesapeake/KV	8.01 ± 0.30 lm	0.24 ± 0.01 rs	19.15 ± 0.72 kl

[a] Values marked by the different letters are statistically different ($P < 0.05$).

3.4.2. Antioxidant Properties of Soft Wheat Bran Samples

HOSC values varied from 38.06 μmol TE/g for the Shirley wheat bran from Wye location to 91.25 μmol TE/g for the USG3665 bran from Poplar Hill location under the experimental conditions used (**Figure 3.3**). USG3665 wheat bran cultivar had higher HOSC than its nine counterparts grown in Poplar Hill and Wye locations, indicating that wheat genotype might alter hydroxyl radical scavenging capacity.

The relative DPPH$^{\bullet}$ radical scavenging capacity (RDSC) ranged from 1.78 and 3.50 μmol TE/g bran among the genotypes grown at the four locations (**Figure 3.4**). Additionally, bran samples of the ten wheat cultivars from Clarksville location had higher or the same RDSC, compared to their counterparts grown in the other three locations. It indicated that growing location may affect the DPPH$^{\bullet}$ scavenging capacity of soft wheat bran samples.

The highest ORAC value in the tested wheat bran samples was 72.74 μmol TE/g for USG3555 bran from Keedysville location, while the lowest ORAC value was 31.47 μmol TE/g observed in the Shirley wheat bran from Wye location (Figure 3.5). These results were in agreement to the observations for Maryland-grown soft winter wheat and bran samples of the two hard winter wheat cultivars grown in the two Colorado locations (Moore et al., 2005; Zhou, Yin, & Yu, 2005). In addition, the USG3555 wheat bran had higher or same ORAC than its nine counterparts grown in Clarksville and Keedysville locations. These results demonstrated that wheat genotype may have possible effect on oxygen radical scavenging ability of soft wheat.

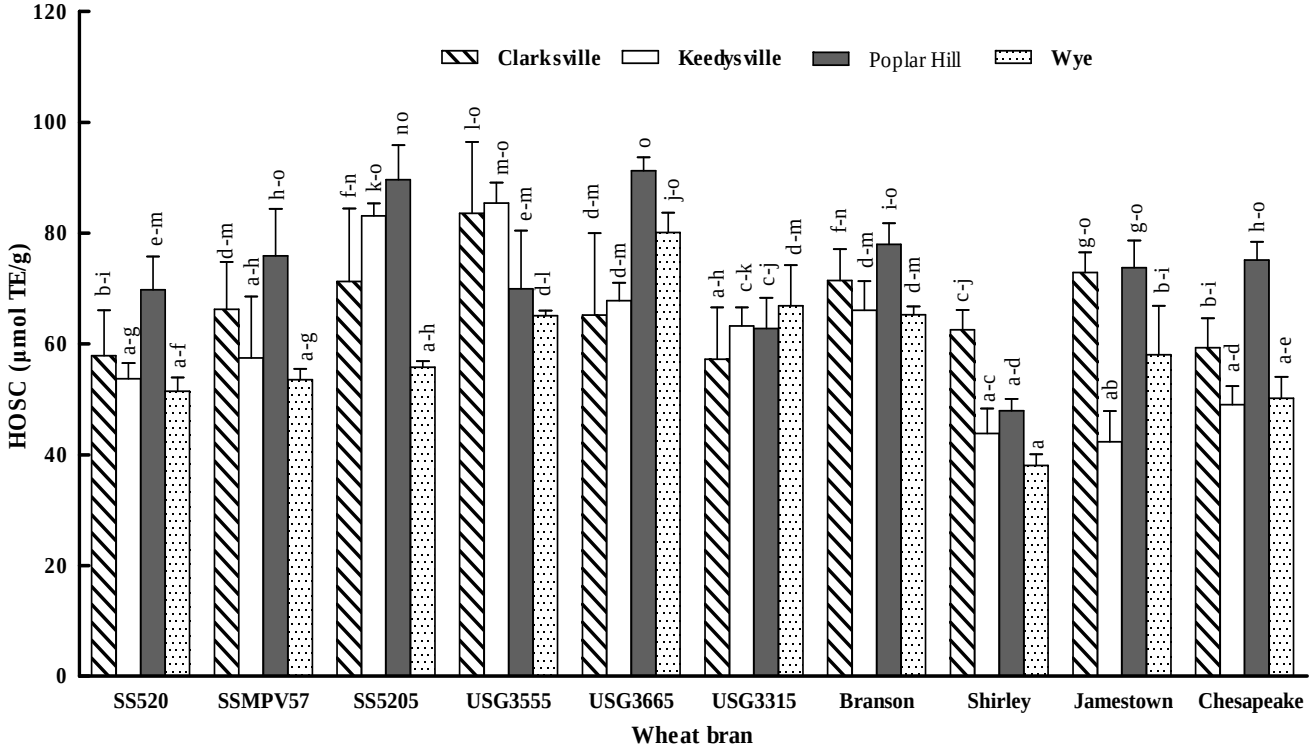

Figure 3.3. Hydroxyl radical (HO•) scavenging capacity (HOSC) of bran samples of soft winter wheat extracts.
SS520, SSMPV57, SS5205, USG3555, USG3665, USG3315, Branson, Shirley, Jamestown, and Chesapeake are ten soft winter wheat cultivars. Clarksville, Keedysville, Poplar Hill, and Wye represent the growing locations. HOSC stands for hydroxyl radical scavenging capacity. Data are expressed as µmol Trolox equivalent (TE)/g of wheat bran. Vertical bars represent the mean of three replicate plots ± SD (n = 3). Values marked by the different letters are statistically different ($P < 0.05$).

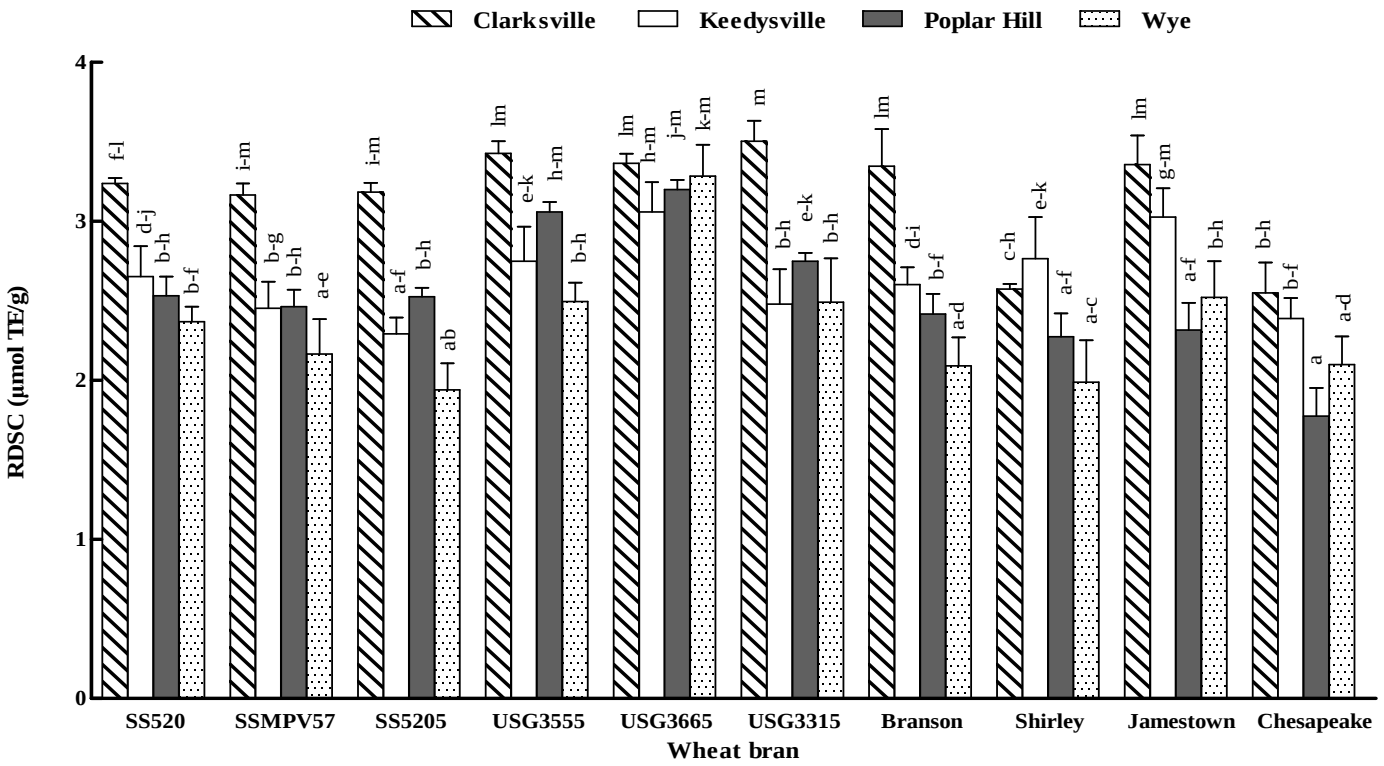

Figure 3.4. Relative DPPH$^{\bullet}$ radical scavenging capacity (RDSC) of bran samples of soft winter wheat extracts.
SS520, SSMPV57, SS5205, USG3555, USG3665, USG3315, Branson, Shirley, Jamestown, and Chesapeake are ten soft winter wheat cultivars. Clarksville, Keedysville, Poplar Hill, and Wye represent the growing locations. RDSC stands for DPPH$^{\bullet}$ scavenging capacity. Data are expressed as µmol Trolox equivalent (TE)/g of wheat bran. Vertical bars represent the mean of three replicate plots ± SD (n = 3). Values marked by the different letters are statistically different ($P < 0.05$).

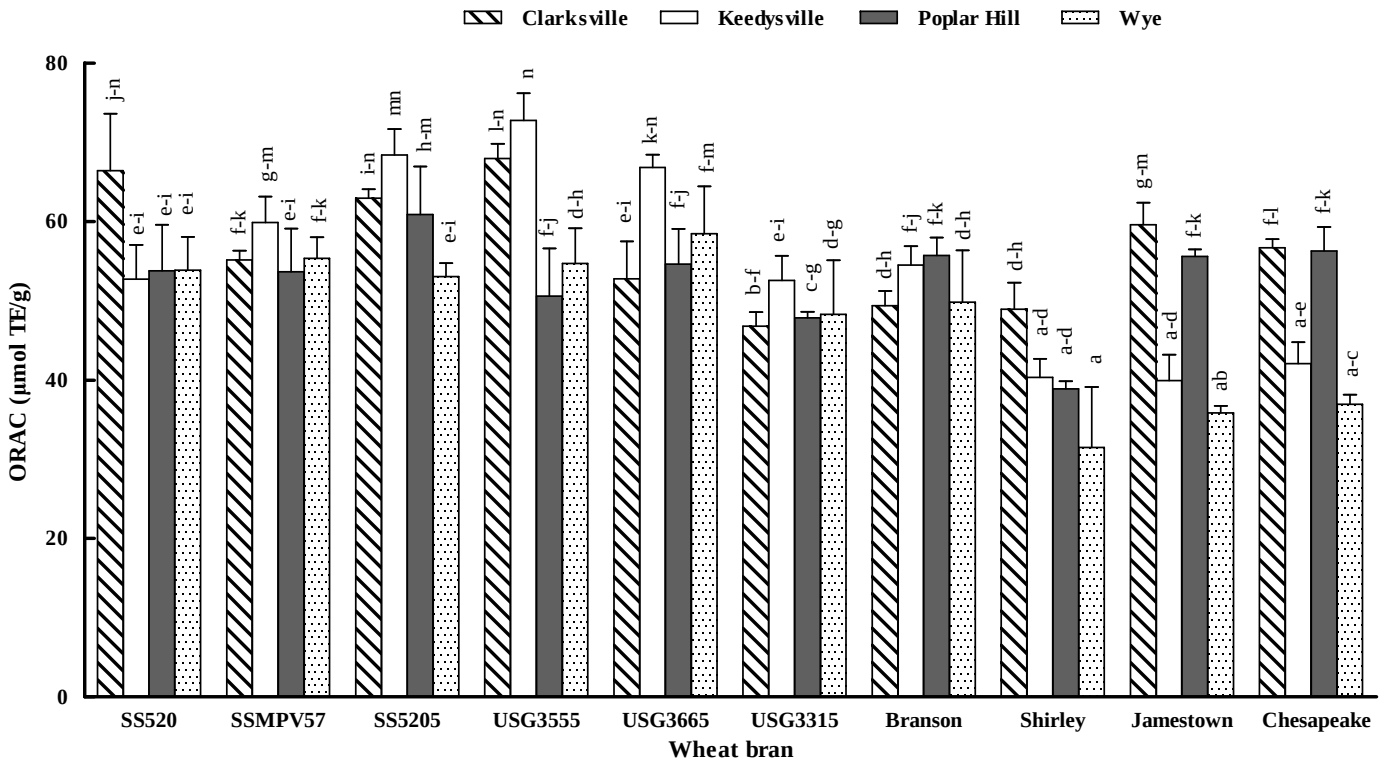

Figure 3.5. Oxygen radical absorbance capacity (ORAC) of bran samples of soft winter wheat extracts.
SS520, SSMPV57, SS5205, USG3555, USG3665, USG3315, Branson, Shirley, Jamestown, and Chesapeake are ten soft winter wheat cultivars. Clarksville, Keedysville, Poplar Hill, and Wye indicate growing location. Abbreviations: ORAC, oxygen radical absorbance capacity. Data are expressed as µmol Trolox equivalent (TE)/g of wheat bran. Vertical bars represent the mean of three replicate plots ± SD (n = 3). Values marked by the different letters are statistically different ($P < 0.05$).

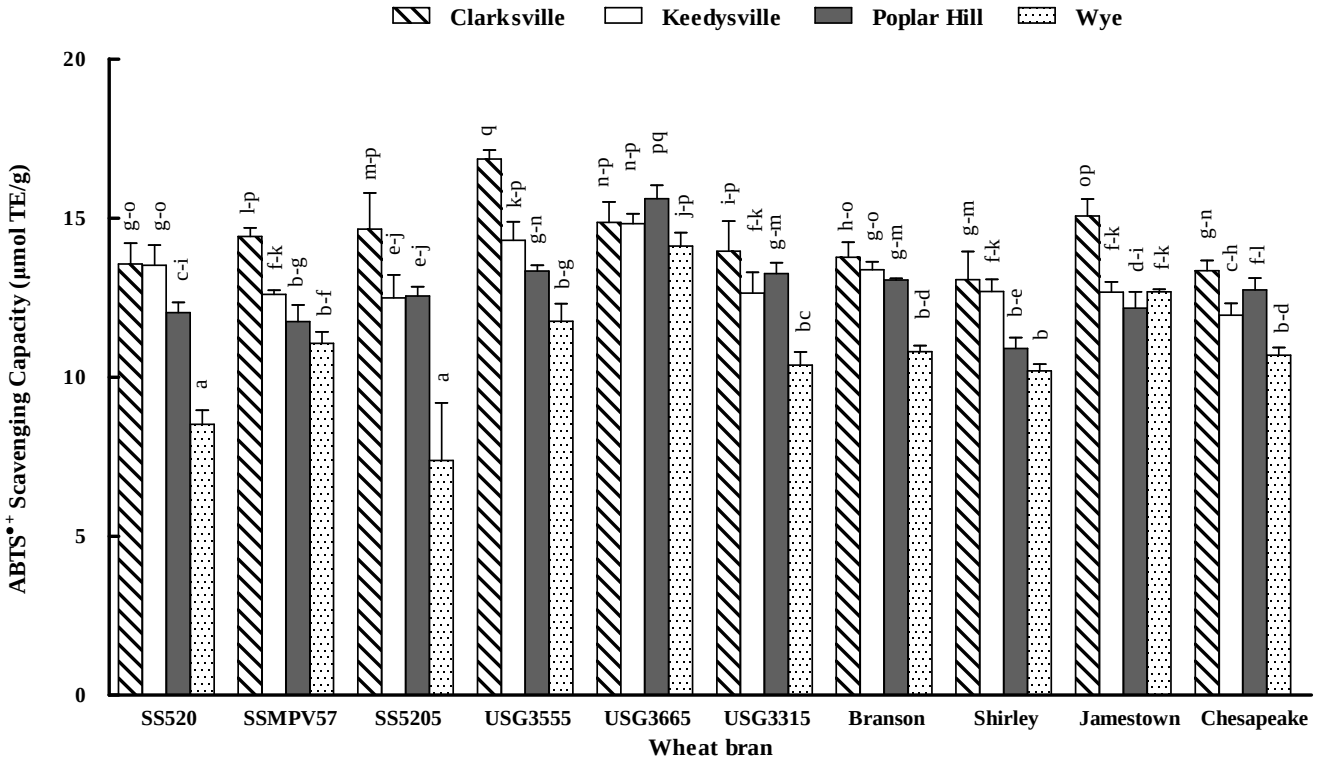

Figure 3.6. ABTS$^{\bullet+}$ scavenging capacity of bran samples of soft winter wheat extracts.
SS520, SSMPV57, SS5205, USG3555, USG3665, USG3315, Branson, Shirley, Jamestown, and Chesapeake are ten soft winter wheat cultivars. Clarksville, Keedysville, Poplar Hill, and Wye indicate growing location. Abbreviation: ABTS$^{\bullet+}$, ABTS$^{\bullet+}$ scavenging capacity. Data are expressed as µmol Trolox equivalent (TE)/g of wheat bran. Vertical bars represent the mean of three replicate plots ± SD (n = 3). Values marked by the different letters are statistically different ($P < 0.05$).

The $ABTS^{\bullet+}$ scavenging capacity ranged from 7.37 and 16.85 μmol TE/g bran among the soft wheat cultivars grown at all four locations (**Figure 3.6**). These data were lower than 17.99-18.85 μmol TE/g for the Colorado hard wheat bran grown in two locations (Zhou, Yin, & Yu, 2005). In addition, a trend in environment effect was shown that all ten wheat cultivars from Clarksville location had higher or same $ABTS^{\bullet+}$ scavenging capacity, compared to the rest samples grown in the other three locations. This suggested that growing environment may affect the $ABTS^{\bullet+}$ scavenging capacity of forty wheat bran samples.

Taken together, this study confirmed the previous findings that soft wheat brans have significant antioxidant properties. These data also indicated that both wheat cultivar and growing environment might have significant influence on the antioxidant properties of soft winter wheat bran (Beta, Nam, Dexter, & Sapirstein, 2005; Zhou, Su, & Yu, 2004a; Zhou & Yu, 2004c).

3.4.3. Effects of Genotype (G), Environment (E), and Their Interaction (G × E) on Soft Wheat Bran Phytochemical Composition and Antioxidant Property

The largest proportion of variation for ORAC was attributed to G (51.50%, $P < 0.001$) (**Table 3.4**). This is in contrast to the observation that E accounted for the most variation for ORAC in three hard winter wheat bran samples grown in five locations ($P < 0.001$) (Moore, Liu, Zhou, & Yu, 2006a) and in soft winter wheat flours (Lv et al., 2013). HOSC of the soft wheat bran was more affected by G (46.90%, $P < 0.001$) (**Table 3.4**), which was different to the study of Lv et al (2013) that E contributed the highest proportion of total variance for HOSC in soft wheat flours (75.78%, $P < 0.001$).

The effect of E accounted for the majority of variance in α-tocopherol, δ-tocopherol, and total tocopherols, at 71.02%, 71.35%, and 71.87%, respectively ($P < 0.001$) (**Table 3.4**). The results from the present study differed from the findings of Lv et al. (2013) that G × E contributed to the highest proportion of variation for α-tocopherol and total tocopherols in soft winter wheat flour samples (71.53%, $P < 0.001$; 71.55%, $P < 0.001$, respectively). The largest proportion of variation in RDSC was attributed to E (45.55%, $P < 0.001$) (**Table 3.4**). This differed from the findings of Moore, Liu, Zhou and Yu (2006a) that G contributed to the highest proportion of variance for $DPPH^{\bullet}$ scavenging capacity in bran samples of the twenty hard winter wheat cultivars grown in two Colorado locations (85.78%, $P < 0.001$) as well as in three hard winter bran samples from five Colorado locations (88.58%, $P < 0.001$. In addition, E contributed the highest proportion of variance in $ABTS^{\bullet+}$ scavenging capacity (50.54%, $P < 0.001$) (**Table 3.4**). This agreed with a previous study that showed E might play the most important role in determining $ABTS^{\bullet+}$ scavenging capacity for hard winter wheat bran samples grown in Colorado (60.07%, $P < 0.001$) and Maryland-grown soft winter wheat flours (91.05%, $P < 0.001$) (Moore, Liu, Zhou, & Yu, 2006a; Lv et al., 2013). E contributed the highest proportion of total variance (43.07%, $P < 0.001$) for TPC (**Table 3.4**). This was consistent to the observation of Moore, Liu, Zhou, & Yu (2006a) that E accounted for most of the variation in TPC (79.54%, $P < 0.001$) for three hard winter wheat brans from five Colorado growing locations and for bran samples of the twenty hard winter wheat cultivars grown in two Colorado locations (68.32%, $P < 0.001$). In addition, Mopfu, Sapirstein, & Beta (2006) reported significant variation in TPC of the six wheat cultivars grown in four Canadian locations by environment (57.86%, $P < 0.001$), which was in agreement with our findings. The largest proportion of variation in total soluble ferulic acid

content was attributed to E (40.44%, $P < 0.001$) (**Table 3.4**), agreeing to that E accounted for most of the variation in ferulic acid content (56.99%, $P < 0.001$) for bran samples of the twenty hard winter wheat cultivars in two locations (Moore, Liu, Zhou, & Yu, 2006a). The observation of Mopfu, Sapirstein, & Beta (2006) also showed that E accounted for the majority of variance in ferulic acid content (56.57%, $P < 0.001$) for six hard spring wheat cultivars grown in four Canadian locations.

G × E showed the strongest influences of 46.73, 45.42, 49.11 and 46.17 % ($P < 0.001$), respectively, on lutein, zeaxanthin, β–carotene and total carotenoid contents (**Table 3.4**). Our study differs from the findings of Lv et al. (2013) that E contributed to the highest proportion of variance for lutein, zeaxanthin, β–carotene and total carotenoid contents in soft winter wheat flour samples. To our knowledge, this is the first report on how genotype and environment may alter carotenoid profile in soft wheat bran samples.

Table 3.4. Effect of genotype (G), environment (E), and G × E interaction on wheat bran composition and antioxidant properties for bran samples of the ten soft winter wheat cultivars grown at four locations [a]

	G (%)	E (%)	G × E (%)
Lutein	16.42***	36.85***	46.73***
Zeaxanthin	17.43***	37.15***	45.42***
β-Carotene	5.37***	45.52***	49.11***
Total Caros	16.65***	37.18***	46.17***
α-Tocopherol	15.24***	71.02***	13.74***
δ-Tocopherol	9.71***	71.35***	18.94***
Total Tocos	14.63***	71.87***	13.50***
TSFA	32.88***	40.44***	26.68***
TPC	36.97***	43.07***	19.96***
ORAC	51.50***	13.30***	35.20***
HOSC	46.90***	20.07***	33.03***
$ABTS^{\bullet+}$	28.79***	50.54***	20.67***
RDSC	36.55***	45.55***	17.90***

[a] Abbreviations: TSFA, total soluble ferulic acid; TPC, total phenolic content; ORAC, oxygen radical absorbance capacity; HOSC, hydroxyl radical scavenging capacity; $ABTS^{\bullet+}$, $ABTS^{\bullet+}$ scavenging capacity; RDSC, $DPPH^{\bullet}$ scavenging capacity; Total Caros, total carotenoids; Total Tocos, total tocopherols; ***, $P < 0.001$. Values without asterisks are not significant at $P < 0.05$. Effects of genotype, environment, and genotype × environment on wheat bran composition and antioxidant properties are expressed as percent of total mean square.

3.4.4. Effect of Individual Environmental Factors on Phytochemical Compositions and Antioxidant Properties of Soft Wheat Bran

As the present results indicate that E may be a significant factor affecting individual antioxidant properties and phytochemical compositions for the ten soft winter wheat brans grown in four Maryland locations, it would be interesting to determine which individual environmental factors acted as the major contributors to the environmental variation. Precipitation, and average low, average high and overall

average temperatures were tested as the possible factors. However, soil compositions were not studied in this research.

To date, there was no report on correlations of the environmental factors with total soluble ferulic acid, zeaxanthin, β-carotene and total carotenoids contents in wheat bran. In the current study, total soluble ferulic acid, β-carotene and total carotenoid contents of soft wheat bran samples were positively correlated with precipitation, average low and high, overall average temperatures ($P < 0.01$) (**Table 3.5**). In addition, zeaxanthin content was positively correlated with the average low temperature ($r = 0.491$, $P < 0.05$) and overall average temperature ($r = 0.437$, $P < 0.01$) (**Table 3.5**).

The TPC of soft winter wheat bran had negative correlations with average low temperature ($r = -0.519$, $P < 0.01$) and overall average temperature ($r = -0.427$, $P < 0.01$) (**Table 3.5**). Lutein content was positively correlated with precipitation ($r = 0.318$, $P < 0.05$), average low temperature ($r = 0.510$, $P < 0.01$), and overall average temperature ($r = 0.481$, $P < 0.01$). In addition, α-tocopherol, δ-tocopherol, and total tocopherols were positively correlated with precipitation ($P < 0.05$) and strong positive correlations with all individual temperature factors ($P < 0.01$) (**Table 3.5**). This might be partially supported by the observation of Shewry et al. (2010) that tocopherols of 26 wheat cultivars grown in six site × year combinations showed strong positive correlations with the average temperature between heading and harvest. There were negative correlations between HOSC and all tested environmental factors ($P < 0.05$). In addition, ORAC had negative correlations with precipitation ($r = -0.313$, $P < 0.05$) and average high temperature ($r = -0.322$, $P < 0.05$), which was consistent to the results from our recent study for soft winter wheat flour samples (Lv et al.,

2013). Furthermore, RDSC was negatively correlated with average low temperature (r = -0.512, $P < 0.01$) and overall average temperature (r = -0.341, $P < 0.05$) (**Table 3.5)**.

$ABTS^{\bullet+}$ scavenging capacity was not correlated with any environmental factor. This was not in agreement with the previous observation of Lv et al. (2013) that there were correlations between $ABTS^{\bullet+}$ scavenging capacity and temperature stress in flour samples of the ten soft winter wheat cultivars grown in four Maryland locations.

Table 3.5. Correlation between antioxidant properties, phytochemical compositions, and weather conditions [a]

	Precipitation	Ave. low temp	Ave. high temp	Overall ave. temp
$ABTS^{\bullet+}$	-0.168	0.205	-0.220	0.009
HOSC	-0.377*	-0.378*	-0.372*	-0.417**
ORAC	-0.313*	-0.125	-0.322*	-0.211
RDSC	-0.096	-0.512**	-0.040	-0.341*
Lutein	0.318*	0.510**	0.297	0.481**
Zeaxanthin	0.249	0.491**	0.224	0.437**
β-Carotene	0.607***	0.645***	0.587***	0.674***
Total Caros	0.595***	0.537***	0.574***	0.572***
α-Tocopherol	0.710***	0.640***	0.710***	0.747***
δ-Tocopherol	0.846***	0.649***	0.845**	0.783***
Total Tocos	0.498**	0.746***	0.442**	0.646***
TSFA	0.637***	0.522**	0.633***	0.610***
TPC	-0.194	-0.519**	-0.160	-0.427**

[a] Abbreviations: Ave. low temp, average low temperature; Ave. high temp, average high temperature; Overall ave. temp, overall average temperature; $ABTS^{\bullet+}$, $ABTS^{\bullet+}$ scavenging capacity; HOSC, hydroxyl radical scavenging capacity; ORAC, oxygen radical absorbance capacity; RDSC, $DPPH^{\bullet}$ scavenging capacity; Total Caros, total carotenoids; Total Tocos, total tocopherols; TSFA, total soluble ferulic acid; TPC, total phenolic content; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Values without asterisks are not significant at $P < 0.05$. Data are expressed as Pearson correlation coefficients (r value).

3.4.5. Intercorrelations between Antioxidant Properties and Phytochemical Compositions

Pearson correlation coefficients between individual wheat bran composition and antioxidant property were shown in **Table 3.6**. The total soluble ferulic acid content was positively correlated with β-carotene, total carotenoids, α-tocopherol and δ-

tocopherol, respectively ($P < 0.05$), but not correlated with any antioxidant property. However, Moore, Liu, Zhou, & Yu (2006a) reported that ferulic acids had positive correlations with TPC and $ABTS^{\bullet+}$ scavenging capacity for the twenty wheat bran samples from two locations ($P < 0.01$). Total tocopherols had negative correlations with ORAC and RDSC ($P < 0.05$), whereas α-tocopherol was negatively correlated with all antioxidant properties except $DPPH^{\bullet}$ scavenging capacity and positively correlated with all individual bran compositions. In addition, lutein was negatively correlated with RDSC ($r = -0.329$, $P < 0.05$). These data might indicate that α-tocopherol and lutein as major components in wheat bran may be highly heritable in soft winter wheat bran and wheat that contained higher levels of these beneficial components might be produced through genetic manipulation.

Correlation analysis also detected positive correlation between TPC and RDSC ($r = 0.623$, $P < 0.001$). This was consistent to the previous observation by Zhou, Su, & Yu (2004a) for the seven wheat bran samples from four different countries. Among antioxidant properties, ORAC was positively correlated with $ABTS^{\bullet+}$ scavenging capacity ($r = 0.329$, $P < 0.05$) and HOSC ($r = 0.500$, $P < 0.01$). This was consistent to the observation of Lv et al. (2013) for soft winter wheat flour samples. In addition, Moore, Liu, Zhou, & Yu (2006a) found that ORAC was positively correlated with $ABTS^{\bullet+}$ scavenging capacity in three hard wheat bran samples grown in five Colorado locations. The data indicated that ORAC of soft winter wheat bran might be a good indicator for both $ABTS^{\bullet+}$ and hydroxyl radical scavenging capacities.

Table 3.6. Correlation between phytochemical compositions and antioxidant properties of bran samples of the ten soft winter wheat cultivars grown at four locations [a]

	ORAC	$ABTS^{\bullet+}$	RDSC	HOSC	Lutein	Zeaxanthin	β-Carotene	Total Caros	α-Toco	δ-Toco	Total Tocos	TSFA
$ABTS^{\bullet+}$	0.329*											
RDSC	0.065	-0.186										
HOSC	0.500**	0.322*	0.287									
Lutein	0.005	-0.033	-0.329*	-0.176								
Zeaxanthin	0.065	0.019	-0.326*	-0.191	0.966***							
β-Carotene	-0.178	-0.032	-0.231	-0.352*	0.327*	0.312*						
Total Caros	-0.040	-0.004	-0.252	-0.215	0.029	0.019	0.201					
α-Toco	-0.458**	-0.371*	-0.256	-0.597***	0.433**	0.373*	0.528***	0.332*				
δ-Toco	-0.213	-0.102	-0.029	-0.244	0.372*	0.308	0.396*	0.458*	0.703***			
Total Tocos	-0.369*	0.281	-0.597***	-0.311	0.285	0.270	0.361*	0.488**	0.355*	0.387*		
TSFA	-0.048	0.032	0.161	-0.091	0.268	0.236	0.451**	0.329*	0.414**	0.632***	0.312	
TPC	-0.058	0.060	0.623***	0.612***	-0.536***	-0.555***	-0.250	-0.210	-0.445**	-0.130	-0.360*	-0.042

[a] Abbreviations: $ABTS^{\bullet+}$, $ABTS^{\bullet+}$ scavenging capacity; HOSC, hydroxyl radical scavenging capacity; ORAC, oxygen radical absorbance capacity; RDSC, $DPPH^{\bullet}$ scavenging capacity; Total Caros, total carotenoids; Total Tocos, total tocopherols; α-Toco, α-tocopherol; δ-Toco, δ-tocopherol; TSFA, total soluble ferulic acid; TPC, total phenolic content; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Values without asterisks are not significant at $P < 0.05$.

3.5 Conclusions

In summary, high levels of variability for selected health beneficial components and antioxidant properties of soft winter wheat brans controlled by E, G and G ×E were observed. E had a strong impact on α-tocopherol, δ-tocopherol, total tocopherols, total phenolic content (TPC), total soluble ferulic acid, and $ABTS^{\bullet+}$ cation and $DPPH^{\bullet}$ radical scavenging capacities ($P < 0.001$), while lutein, zeaxanthin, β-carotene, and total carotenoid contents of soft wheat brans were more affected by G × E interaction ($P < 0.001$). Peroxyl (ORAC) and hydroxyl (HOSC) radical scavenging capacities were more affected by G ($P < 0.001$). In general, E played a larger role in variations of individual phytochemical component and antioxidant property of soft wheat bran than did G and G ×E, agreeing with the previous studies (Lv et al., 2013; Moore, Liu, Zhou, & Yu, 2006a). Each soft wheat bran component or antioxidant property might respond to individual environmental factors differently. Among the soft wheat bran studied, there was not one particular genotype or environment that produced outstanding levels of all health components. However, it may be possible to choose the ideal wheat cultivars and growing locations for an enhanced level of a specific component. In addition, this study along with our previous studies showed that different wheat classes (soft and hard) and different wheat fractions (bran and flour) may respond to the effects of G, E, and G × E differently. Based on the results of this study, it might be possible for wheat breeders, growers, and grain traders to select optimal environment and genotype to improve the levels of selected health components and antioxidant properties of soft winter wheat.

Chapter 4: Effect of processing on phytochemical profile and antiproliferative activity of dough and bread fractions made from refined and whole wheat flours

4.1 Abstract

Phytochemicals profile (phenolic acids, carotenoids, and tocopherols) and antiproliferative properties of bread processing fractions, including the dough, crumb, and upper crust made from refined wheat and whole wheat flours were analyzed using two wheat cultivars, 'Louise' (soft white) and 'Macon' (hard white). Ferulic acid, lutein and α-tocopherol were the predominant phenolic acid, carotenoid, and tocopherol, respectively, extracted from all fractions. The quantities of phenolic acids, carotenoids, and tocopherols were significantly higher in all fractions made from whole wheat flour than their corresponding refined wheat flour fractions. The concentrations of phenolic acids (soluble and insoluble bound) in the upper crust of refined and whole wheat breads made from both wheat cultivars (Louise and Macon) were higher than their dough fractions, respectively. In addition, the dough of whole wheat had higher levels of tocopherols and carotenoids as compared to crumb and upper crust, suggesting some possible degradation of tocopherols and carotenoids during baking. The antiproliferative activity of whole wheat bread extracts against HT-29 cancer cells was positively correlated with total phenolic acids, but showed no correlations with total carotenoids and total tocopherols content. The results indicate that baking reduces the concentrations of carotenoids and tocopherols, however, the upper crust fraction had significant higher

levels of phenolic acids than in the dough and crumb fractions, suggesting that total phenolic acids content might not decrease during baking.

4.2 Introduction

There is growing consumer awareness of the health consequences of dietary choices. This awareness is based, in part, on a growing body of information on bioactive phytochemicals such as carotenoids, tocopherols, phenolic compounds, and other secondary metabolites that are commonly present in fruits, vegetables, and grains. Wheat accounts for approximately 71% of total grain consumption in the USA in 2005 (USDA, 2005). In addition to being an important source of carbohydrates, wheat also provides dietary fiber, protein, minerals, vitamins, and other bioactive compounds (Slavin, 2004; Piironen et al., 2009).

In recent years, there has been renewed interests in whole grain foods as numerous epidemiological and clinical studies have indicated that consumption of whole grain foods can significantly reduce the risk of numerous chronic health conditions such as type 2 diabetes, cardiovascular disease, and colon cancer (Slavin, 2004; Jones, 2006). Initially, it was hypothesized that the health beneficial effect of whole grain was primarily due to its high fiber content (Trowell, 1972). However, recent epidemiological studies suggests that the beneficial effect of whole grain may arise from the combined action of several components such as fiber, vitamins, and phenolic phytochemicals (Anderson, 2004; Slavin, 2004; Slavin, Marquart, & Jacobs, 2000; Piironen et al., 2009).

There have been few studies reported in the literature on changes in the fiber and phytochemicals during bread making. Hensen et al. (2002) investigated changes in the

dietary fiber, phenolic acids, and activity of endogenous enzymes during making of rye bread. The authors analyzed monomeric and dimeric phenolic acids in rye whole meal flour, dough, and bread. The results showed that the content of total ester-bound phenolic acids and ferulic acid dehydrodimers decreased from 1575 µg/g in the wholemeal to 1472 µg/g in rye bread. The antioxidant activity of rye bread fractions was analyzed by three different procedures namely, Folin-Ciocalteu, oxygen radical antioxidant capacity (ORAC), and Trolox equivalent antioxidant capacity (TEAC) by Mickalska, Amigo-Benavent, Zielinski, & Del Castillo (2008). The authors observed an increase in antioxidant activity during baking. In another recent study, Moore, Luther, Cheng, & Yu (2009), investigated the effect of baking conditions (time and temperature), dough fermentation, and bran particle size on antioxidant properties of pizza crusts made from two whole wheat cultivars (Trego and Lakin).The authors observed that increasing baking time and temperature improved the antioxidant activity while no significant change in antioxidant activity was observed with variations in bran particle size and fermentation time. The changes in the tocopherols and carotenoids content during the production of bread, water biscuits, and pasta from wheat flours were recently investigated (Hidalgo & Brandolini, 2010a; Hidalgo, Brandolini, & Pompei, 2010b). The total carotenoids and tocopherols content decreased during processing. In a recent study, Abdel-Aal and Rabalski (2013) investigated the effect of baking on free and bound phenolic acids in whole grain bakery products. The authors observed that baking resulted in increased free phenolic acids in all three products (bread, cookies, and muffins) while the bound phenolic acids decreased in bread and slightly changed in cookies and muffins.

In the present study, we investigated the effect of bread-making on three classes of phytochemicals, namely, phenolic acids, tocopherols, and carotenoids. Two wheat cultivars, 'Louise' (soft white spring wheat) and 'Macon' (hard white spring wheat) were selected for comparison in this study. Both refined wheat (RF) and whole wheat (WW) flours were separately used for bread-making. Phytochemicals were analyzed in dough, crumb, and upper crust fractions. In addition, the antiproliferative activity of extracts from all three fractions was also investigated using HT-29 human colon cancer cells.

4.3 Materials and Methods

4.3.1. Wheat and Bread Samples

Spring wheat cultivars, 'Louise' (soft white) and 'Macon' (hard white) were grown as previously described (Whent et al., 2012). Grain was tempered and milled to approximately 70% extraction (refined wheat flour) on a Buhler MLU-202 flour mill. Particle size of bran and shorts mill streams were reduced with a pin mill so that ≥ 70% passed through a 180 µm sieve; reduced fractions were then blended with the refined wheat flour to produce whole wheat flour. Flour samples were stored at -20 ^{o}C prior to processing.

Bread was prepared following the 100-gram straight-dough bread-making method (AACCI Approved Method 10-10B). A dough sample was frozen immediately after mixing. After baking, the loaves were divided into the following three components: 'upper crust', which represented that part of the loaf exposed directly to oven temperatures, 'bottom crust' (not analyzed here), representing that part of the crust in contact with the loaf pan, and 'crumb', which was everything except both crust fractions.

The crust components were scraped to remove the adhering crumb component. Dough, crumb, and upper crust components were lyophilized and stored at -20 ^{o}C. Dried samples were broken up with a mortar and pestle and ground in a Tecator Cemotec 1090 burr mill set to the finest setting. Dough, crumb, and upper crust fractions were ground to a particle size of 40-mesh using a Micromill manufactured by Bel Art Products (Pequannock, NJ, USA).

4.3.2. Chemicals

Tocopherols (α-, δ-, and γ-), ascorbic acid, and β-carotene were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lutein and zeaxanthin were obtained from Indofine Chemical Co. Inc. (Hillsborough, NJ, USA). All other chemicals and solvents were of the analytical reagent grade and used without further purification. Human colon cell line HT-29 was purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). McCoy's 5A medium was obtained from Gibco Life Technology (Grand Island, NY, USA) and antibiotic/antimycotic solution was purchased from Gibco Invitrogen Corporation (Carlsbad, CA, USA).

4.3.3. Phenolic Acid Composition

Soluble free, soluble conjugated and insoluble bound phenolic acids were extracted according to the method described previously (Moore et al., 2005). Ground dough, crumb, or upper crust fractions were extracted with acetone/methanol/water (7:7:6, v/v/v) first to obtain two fractions, the soluble supernatant and the residue. The residue was hydrolyzed with sodium hydroxide, and then extracted with ethyl ether and ethyl acetate (1:1, v/v) for

analysis of insoluble bound phenolic acids. Soluble free and conjugated phenolics in the supernatants were separated under acidic conditions (pH = 2) and extracted with ethyl ether and ethyl acetate (1:1, v/v). After evaporating the organic phase under nitrogen, each extract was re-dissolved in methanol and filtered through a 0.45 µm membrane filter, then analyzed by the HPLC. HPLC separation of phenolic acids was accomplished using a Shimadzu LC-20AD with an autosampler, a Phenomenex C18 column (4.6 mm i.d. × 250 mm, 5 µm particle size) at 25 ^{o}C and a UV-VIS detector. Briefly, the HPLC elution program was as follows: mobile phase A consisted of acetic acid: H_2O (2:98, v/v) and mobile phase B consisted of acetic acid: acetonitrile: H_2O (2:30:68, v/v/v). Elution was programmed from 10 to 100% B in 42 min with a flow rate of 1.0 mL/min. Injection volume was 10 µL. The individual phenolic acids were identified by comparing UV spectral data and retention time with authentic commercial standards. Quantification was achieved by measuring the area under the peak of external standards. Results were expressed as µg/g or µmol/100 g of dough, crumb, or the upper crust on a dry weight basis.

4.3.4. Carotenoid Composition

Two hundred mg of each ground dough, crumb, or upper crust sample was extracted with 10 mL of methanol: tetrahydrofuran (1:1, v/v) for 15 hours at ambient temperature. The resulting extraction mixture was centrifuged at 2,000 rpm. The solvent was decanted and then dried under N_2. The residue was re-dissolved in 2 mL of methanol: acetonitrile: iso-propanol (54:44:2, v/v/v), and filtered through a 0.45 µm membrane filter. HPLC separation of carotenoids was accomplished according to a previously described

laboratory protocol using water as solvent A and methanol: acetonitrile: iso-propanol (54:44:2, v/v/v) as solvent B. The gradient procedure was as follows: 1) the gradient was linear from 95% to 99% of solvent B, and the flow rate was 1 mL/min in the first 10 min, 2) 99% of solvent B and a flow rate of 1 mL/min for 10 min, and 3) the gradient was linear from 99% to 95% of solvent B for the last 5 min (Moore et al., 2005). Twenty µL of each standard or sample was injected. Detection wavelength was at 450 nm. A standard curve was developed using authentic standards, and the area under the peak was used for quantification. Results were expressed as µg/g or µmol/100 g of dough, crumb, or the upper crust on a dry weight basis.

4.3.5. Tocopherol Composition

HPLC separation was accomplished using the same HPLC system and column previously mentioned according to a previously described protocol (Zhou, Yin, & Yu, 2005). The tocopherols were separated using an isocratic elution with a a mobile phase 1% solvent A (water) and 99% solvent B (acetonitrile). The flow rate was 1.5 mL/min. Injection volume was 20 µL for each standard or sample. A standard curve was developed from the known standards (α-, δ-, and γ-tocopherol), and peak areas were used for quantification. Results were expressed as µg/g or µmol/100 g of dough, crumb, or the upper crust on a dry weight basis.

4.3.6. Antiproliferative Effects of Selected Bread and Dough Extracts in HT-29 Human Colon Cancer Cells

The antiproliferative effects of whole wheat dough, crumb, and upper crust extracts were tested in HT-29 human colon adenocarcinoma cells according to the method described by Slavin and others (Slavin, Kenworthy, & Yu, 2009). Refined wheat samples were not evaluated for the antiproliferative activity due to their low concentrations of phytochemicals. Each sample (dough, crumb, or upper crust, 0.45 g) was extracted with 4.5 mL 50% acetone overnight at ambient temperature. The supernatant was collected, and the solvent was evaporated using a nitrogen evaporator. The solid residues were re-dissolved in DMSO. Culture medium consisted of McCoy's 5A media supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution.

Cells were plated at 2.5×10^3 cells/well culture medium in 96-well plates. After a 24-hour incubation time, the culture medium was replaced with 100 µL of treatment media containing 0.0 (vehicle), 9.0, 22.5, 45.0 mg of sample (dough, crumb, or upper crust) equivalents/mL of treatment media. All media had a final concentration of 1% Dimethyl sulfoxide (DMSO) (v/v). A culture medium without 1% DMSO (v/v) was also included as a control. Each dough, crumb or upper crust sample was extracted three separate times, and three replicate assays were conducted. Cell proliferation was studied using the ATP-Lite 1 step kit (Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA). Luminescence readings were taken on a Victor3 multi-well plate reader (Perkin Elmer, Turku, Finland) immediately prior to treatment and at 0, 24, 48, 96 hours of treatment. A separate plate was used for each reading. Treatment and control media were replaced every 24 hours until a reading was taken on that plate.

4.3.7. Statistical Analysis

Data were reported as means ± standard deviations (SD) for triplicate treatments. One-way analysis of variance (ANOVA) and Tukey's tests were performed using SPSS (SPSS for Windows, version 10.0.5, SPSS Inc., Chicago, IL, USA). Correlation analyses were performed using a two-tailed Pearson's correlation test. Statistical significance was declared at $P < 0.05$.

4.4 Results and Discussion

4.4.1. Phenolic Acid Composition

Three categories of phenolic acids were analyzed: soluble free, soluble conjugated, and insoluble bound phenolic acids. **Table 4.1** shows the amount of insoluble bound phenolic acids. All three fractions (dough, crumb, and upper crust) of bread made from whole wheat (WW) flour had around tenfold higher insoluble bound total phenolic acids compared to the respective refined (RF) counterparts. Bubbles, Lu, Clydesdale, & Decker (2000) found that whole grain cereal contained threefold higher total phenolic acids than refined wheat cereal. Similar higher antioxidant activity was observed with extracts from wheat-based ready-to-eat breakfast cereals manufactured with high bran or whole grains as compared to the cereals produced from refined wheat (Baublis, Decker, & Clydesdale, 2000). Ferulic acid was the most abundant insoluble bound phenolic acid accounting for about 90% in all three fractions from both wheat cultivars. This was consistent with the previous study by Moore et al. (2005) where insoluble bound ferulic acid accounted for 85% of the total identified insoluble phenolic acids for soft wheat.

In Louise whole wheat bread, the upper crust fraction had the highest total insoluble phenolic acid quantities among all three bread processing fractions, followed by dough and crumb fractions (**Table 4.1**). This was consistent with the findings of Moore, Luther, Cheng, & Yu (2009) where higher levels of insoluble bound ferulic acid were found in baked pizza crust than dough. Mattila, Pihlava, & Hellstrom (2005) also reported that baking did not reduce the concentration of phenolic acids, similar to the results obtained in our current study. Similarly, the dough of the refined wheat bread had comparable or lower levels of insoluble bound ferulic acid and total phenolic acids as compared to their crumb and upper crust counterparts, indicating that baking may increase the levels of insoluble bound ferulic acid and total phenolic acids in crumb and upper crust parts of refined wheat bread. The amount of total insoluble bound total phenolic acids in all three fractions (dough, crumb, and upper crust) investigated in the present study were lower than the total insoluble bound phenolic acids extracted from their respective flour samples reported previously (Whent et al., 2012).

The amounts of two prominent soluble free and conjugated phenolic acids namely, ferulic and *p*-coumaric acids in dough, crumb, and upper crust are shown in **Table 4.2.** As expected, whole wheat dough, crumb, and upper crust samples of both wheat cultivars (Louise and Macon) had higher soluble free ferulic and *p*-coumaric acids than their refined counterparts **(Table 4.2).** The whole wheat dough from Louise and Macon cultivars contained soluble free ferulic acid (2.31-2.41 µg/g) and *p*-coumaric acid (0.34 µg/g), which was consistent with the results described in a previously reported study (soluble free ferulic acid (1.88-1.91 µg/g) and *p*-coumaric acid (0.25-0.27 µg/g)) for Louise and Macon whole wheat flour (Whent et al., 2012). The amount of soluble ferulic,

p-coumaric, and total phenolic acids were higher in crumb and upper crust fractions (only expect for RF Louise crumb fraction) as compared to the dough fraction, indicating that heating influences the release of bound phenolic acids. A similar increase in soluble phenolic acids during processing was reported previously (Piironen, Lampi, Ekholm, Salmenkallio-Marttila, & Liukkonen, 2009; Zielinski, Kozlowska, & Lewczuk, 2001) as well as in a recent study by Abdel-Aal & Rabalski (2013) during baking in all three products (bread, cookies, and muffins).

Table 4.1. Insoluble bound phenolic acids of the crust and crumb parts of breads and their four corresponding dough samples.[a]

Type	Variety	Fraction	Vanillic (µg/g)	Syringic (µg/g)	Caffeic (µg/g)	*p*-Coumaric (µg/g)	Ferulic (µg/g)	Total PAs (µmol/100 g)
RF	Louise	Mixed Dough	1.25cd± 0.29	0.55d±0.09	0.25b±0.01	0.38bc±0.02	29.7d±2.59	16.7d±1.57
RF	Louise	Crumb	1.13d±0.28	0.60cd±0.06	0.18c±0.01	0.37c±0.02	41.1bc±1.91	22.5bc±1.20
RF	Louise	Upper Crust	1.95ab±0.29	0.68bc±0.15	0.35a±0.05	0.48b±0.05	52.9a±6.61	29.2a±3.71
RF	Macon	Mixed Dough	2.20ab±0.38	0.81bc±0.05	0.26b±0.02	0.38bc±0.01	36.0cd±3.45	20.6cd±2.05
RF	Macon	Crumb	1.79bc±0.25	0.85b±0.06	0.23bc±0.01	0.37c±0.02	37.6bc±4.21	21.2bc±2.36
RF	Macon	Upper Crust	2.45a±0.12	1.18a±0.08	0.26b±0.01	0.60a±0.05	43.9b±6.81	25.2b±3.65
WW	Louise	Mixed Dough	9.74a±2.29	6.30c±1.45	1.35bc±0.18	3.16b±0.10	587.0b±52.9	313.9b±29.5
WW	Louise	Crumb	5.43b±1.05	4.21d±0.78	0.73d±0.25	2.40c±0.08	322.0d±5.82	173.0d±4.2
WW	Louise	Upper Crust	9.98a±1.75	6.57c±0.42	3.88a±0.41	4.54a±0.60	654.5a±27.2	351.2a±15.9
WW	Macon	Mixed Dough	9.14a±0.86	8.86a±0.75	1.63b±0.19	2.75bc±0.11	472.9c±48.6	256.0c±26.1
WW	Macon	Crumb	8.11a±1.27	7.14bc±0.66	1.07cd±0.18	1.80d±0.13	433.5c±14.9	233.4c±8.9
WW	Macon	Upper Crust	8.99a±0.85	8.52ab±0.82	1.36bc±0.17	4.23a±0.36	623.8ab±25.7	334.2ab±14.5

[a] Louise (a soft white spring wheat) and Macon (a hard white spring wheat) are two wheat cultivars. RF - refined wheat; WW - whole wheat. Vanillic, Syringic, Caffeic, *p*-Coumaric, Ferulic and Total PAs stand for vanillic, syringic, caffeic, *p*-coumaric, ferulic and total phenolic acids, respectively. Results expressed as µg/g or µmol/100 g of bread (dough, crumb, or upper crust) on a dry sample weight basis. The results were expressed as Mean followed by a letter. Same letter means no statistical significant difference ($P > 0.05$).

Table 4.2. Soluble phenolic acids content of the dough, crumb and crust parts of breads made from refined and whole wheat bread of two wheat cultivars (Louise and Macon).[a]

	p-Coumaric (µg/g)			Ferulic (µg/g)			*p*-Coumaric+ Ferulic (µmol/100 g)		
T+ V+ F	Free	Conjugated	Total	Free	Conjugated	Total	Free	Conjugated	Total
RF L MD	0.13c±0.00	0.61b±0.03	0.74b±0.03	1.47e±0.28	25.62c±2.81	27.09c±3.09	0.84e±0.14	13.57c±1.47	14.40c±1.61
RF L C	0.26b±0.01	0.71b±0.04	0.97b±0.04	1.91d±0.19	17.04d±1.09	18.95d±1.27	1.14d±0.10	9.21d±0.59	10.35d±0.68
RF L UCT	0.38a±0.02	1.68a±0.23	2.05a±0.25	3.67b±0.25	33.32b±2.65	36.99b±2.89	2.12b±0.14	18.18b±1.50	20.30b±1.64
RF M MD	0.14c±0.00	0.72b±0.05	0.86b±0.05	1.00f±0.20	14.72d±3.01	15.72d±3.20	0.60f±0.10	8.02d±1.58	8.62d±1.68
RF M C	0.37ab±0.0	1.72a±0.24	2.09a±0.21	2.82c±0.45	24.83c±2.54	27.65c±2.97	1.68c±0.24	13.83c±1.45	15.51c±1.66
RF M UCT	0.46a±0.03	1.80a±0.19	2.25a±0.19	5.58a±0.49	50.04a±3.11	55.62a±3.60	3.15a±0.27	26.87a±1.71	30.01a±1.97
WW L MD	0.34b±0.02	1.96c±0.21	2.30d±0.21	2.31d±0.19	33.75d±3.20	36.07d±3.39	1.40d±0.11	18.57d±1.76	19.98d±1.87
WW L C	0.39b±0.01	2.18b±0.23	2.58c±0.19	11.50a±0.52	101.98a±11.3	113.5a±11.1	6.16a±0.27	53.85a±5.96	60.01a±6.09
WW LUCT	0.60a±0.02	2.27b±0.24	2.88b±0.28	7.31c±0.49	64.83c±6.02	72.14c±6.52	4.13c±0.26	34.77c±3.25	38.91c±3.53
WW MMD	0.34b±0.01	1.56d±0.18	1.90e±0.16	2.41d±0.22	33.16d±4.29	35.57d±4.51	1.45d±0.12	18.03d±2.32	19.48d±2.42
WW M C	0.60a±0.02	2.52a±0.31	3.13a±0.25	10.32b±0.91	90.13ab±3.19	100.45b±4.0	5.68b±0.48	47.9ab±1.83	53.64b±2.26
WWMUCT	0.57a±0.02	2.51a±0.30	3.10ab±0.3	9.43b±0.82	82.98b±7.01	92.41b±7.83	5.20b±0.43	44.26b±3.79	49.48b±4.21

[a] T – Type; V – Variety; F – Fraction; L - Louise (a soft white spring wheat) and M - Macon (a hard white spring wheat) are two wheat cultivars; MD - Mixed dough; C – Crumb; UCT – Upper crust; RF - refined wheat; WW - whole wheat; Total – Total soluble *p*-coumaric or ferulic acids. *p*-Coumaric and Ferulic stand for *p*-coumaric and ferulic acids, respectively. Results expressed as µg/g or µmol/100 g of bread (upper crust, crumb or mixed dough) on a dry sample weight basis. The results were expressed as Mean followed by the same letter are not significantly different ($P > 0.05$).

4.4.2. Carotenoid Composition

Lutein was the predominant carotenoid in all three fractions (dough, crumb, and upper crust) regardless of wheat cultivar (Louise and Macon) and flour type (refined and whole wheat), agreeing with the previously reported observation by Hidalgo, Brandolini, & Pompei (2010b). The lutein concentration of the whole wheat upper crust and dough fractions were around tenfold higher than the corresponding bread fraction made from refined bread. However, only 3 fold increase in lutein concentration was observed in crumb fraction made from the whole wheat as compared to the refined counterparts.

The lutein concentration (2.37-2.49 µg/g) in a dough fraction of whole wheat bread from two cultivars were similar to those reported by Whent et al. (2012) for whole wheat flour (2.07-2.70 µg/g). However, the lutein concentration (0.12-0.13 µg/g) in the upper crust of refined bread in the present study was lower (0.20-0.30 µg/g) than previously reported by Hidalgo, Brandolini, & Pompei (2010b) **(Table 4.3)**. The lutein concentration (1.92-2.00 µg/g) in the upper crust of bread made from whole wheat was higher than that for Finnish durum wheat bread made from whole wheat flour (0.71 µg/g) (Heinonen, Ollilainen, Linkola, Varo, & Koivistoinen, 1989).

Table 4.3. Carotenoid and tocopherol profile of the dough, crumb and crust parts of breads made from refined and whole wheat bread of two wheat cultivars (Louise and Macon).[a]

Type	Variety	Fraction	Lutein (µg/g)	Zeaxanthin (µg/g)	Total Carotenoids (µmol/100 g)	α-tocopherol (µg/g)	δ-tocopherol (µg/g)	Total Tocopherols (µmol/100 g)
RF	Louise	Mixed Dough	0.12a±0.00	0.11a±0.00	0.04a±0.00	0.79b±0.02	0.13ab±0.00	0.21b±0.01
RF	Louise	Crumb	0.12a±0.00	0.11a±0.00	0.04a±0.00	0.48e±0.01	0.09c±0.00	0.13e±0.00
RF	Louise	Upper Crust	0.12a±0.00	0.11a±0.00	0.04a±0.00	0.58d±0.01	0.09c±0.00	0.16d±0.00
RF	Macon	Mixed Dough	0.13a±0.00	0.12a±0.00	0.04a±0.00	0.95a±0.02	0.14a±0.00	0.25a±0.01
RF	Macon	Crumb	0.13a±0.00	0.12a±0.00	0.04a±0.00	0.49e±0.01	0.11c±0.00	0.14de±0.00
RF	Macon	Upper Crust	0.13a±0.00	0.12a±0.00	0.04a±0.00	0.67c±0.01	0.11bc±0.00	0.18c±0.00
WW	Louise	Mixed Dough	2.37b±0.20	0.51a±0.02	0.51b±0.04	18.99b±0.55	1.34b±0.09	4.72b±0.14
WW	Louise	Crumb	0.48e±0.02	0.24c±0.01	0.12e±0.01	3.45e±0.21	0.54f±0.00	0.93f±0.05
WW	Louise	Upper Crust	0.92d±0.09	0.36b±0.01	0.40d±0.03	12.62d±0.22	0.78d±0.02	3.11d±0.05
WW	Macon	Mixed Dough	2.84a±0.11	0.51a±0.03	0.59a±0.03	21.39a±1.11	1.37a±0.11	5.28a±0.28
WW	Macon	Crumb	0.49e±0.01	0.25c±0.02	0.13e±0.00	3.77e±0.29	0.69e±0.02	1.04e±0.07
WW	Macon	Upper Crust	2.00c±0.11	0.37b±0.02	0.42c±0.02	14.73c±0.18	1.08c±0.03	3.67c±0.05

[a] Louise (a soft white spring wheat) and Macon (a hard white spring wheat) are two wheat cultivars. RF - refined wheat; WW - whole wheat. Results expressed as µg/g or µmol/100 g of bread (upper crust, crumb or dough) on a dry sample weight basis. The results were expressed as Mean followed by the same letter are not significantly different ($P > 0.05$).

In the present study, zeaxanthin was detected in all fractions. These results are different from the observation of Hidalgo, Brandolini, & Pompei (2010b) where no detectable levels of zeaxanthin were observed in soft wheat bread. As shown in **Table 4.3**, the upper crust of whole wheat bread had lower concentrations of lutein, zeaxanthin and total carotenoids than their corresponding dough, but higher than those in the crumb, indicating that carotenoids may be partially degraded during baking. Around 25% decrease in total carotenoids concentration was observed in upper crust as compared to the dough fraction for both wheat cultivars. This is in agreement with the previously reported study where 5-43% decrease in the carotenoids level was observed during pasta processing (Piironen et al., 2009).

4.4.3. Tocopherol Composition

In the present study, α-tocopherol was the predominant tocopherol in all fractions regardless of wheat variety and flour type, agreeing with the observation of Moore et al. (2005). Minor quantity of δ-tocopherol was also detected in all fractions while no δ-tocopherol was analyzed in the study of Moore et al (2005) for soft wheat. The α-tocopherol concentration in whole wheat mixed dough for Louise and Macon cultivars ranged between 18.99-21.39 µg/g. This is in agreement with the previously reported values by Whent et al. (2012) for Louise and Macon whole wheat flour (17.32-20.89 µg/g), respectively. In general, the dough had higher levels of α- and δ-tocopherols than crumb and upper crust regardless of wheat variety and flour type **(Table 4.3)**. These results suggest possible degradation of tocopherols during baking. Around 30% decrease

in total tocopherols concentration was observed in upper crust as compared to the dough fraction for both wheat cultivars. This agreed with the study of Leehardt et al. (2006) where around 30% decrease in total tocopherols concentrations was observed during bread-making. However, these results are different from the findings by Hidalgo & Brandolini (2010a) where no alteration in the tocopherol levels was observed during bread making for einkorn wheat made from refined flour. The total tocopherol concentration of the whole wheat upper crust and dough fractions were around twenty-folds higher than the corresponding bread fraction made from refined bread. However, only six to sevenfold increase in α-tocopherol concentration was observed in crumb fraction made from the whole wheat as compared to the refined counterparts. Total tocopherols were positively correlated with total carotenoids ($r = 0.995$, $P < 0.001$), and total insoluble bound phenolic acids ($r = 0.903$, $P < 0.001$).

4.4.4. Antiproliferative Activity in HT-29 Human Colon Cancer Cells

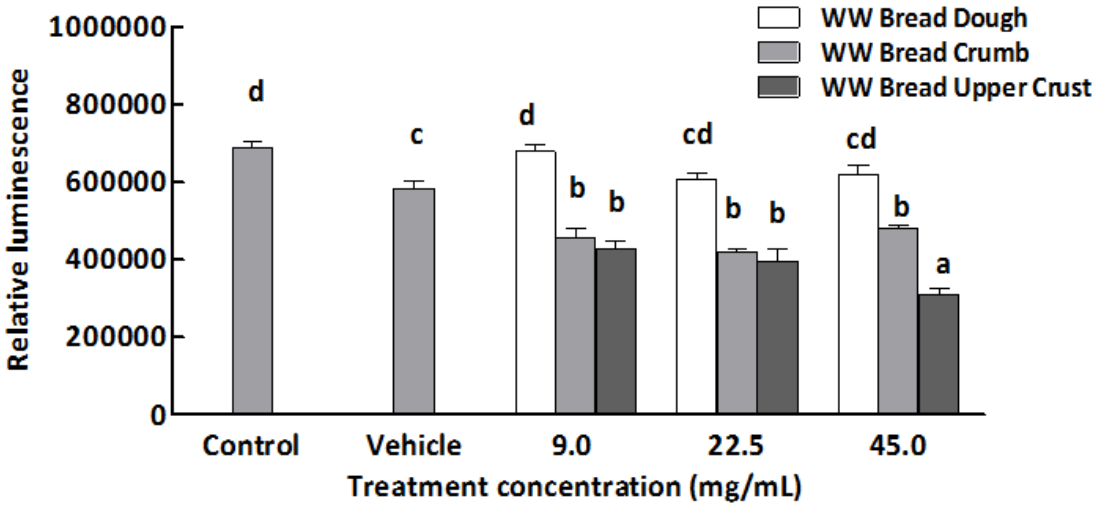

Figure 4.1. Antiproliferative effects of extracts from dough, crumb, and crust made from Louise (soft white wheat cultivar) whole wheat flour, in cultured HT-29 human colon cancer cells.
HT-29 cells (2.5 x 10^3 cells/well) were incubated overnight prior to treatments. Control, Culture medium only; Vehicle, Culture medium with 1% DMSO. Culture media was then removed and treatment media containing the extracts of dough, crumb, and crust were then added at the indicated concentrations and incubated for 96 hrs. Relative luminescence is proportional to the number of viable cells. Values were based on triplicate tests, with mean ± SD shown (n = 3). The statistical significance of samples is indicated using letters and results labeled by the same letter are not significantly different ($P > 0.05$).

Colon cancer is the third leading cause of death among men and the second leading cause of death in women (Jemal et al., 2011). Ferulic acid, the predominant phenolic acid found in wheat grain, has shown antiproliferative effects on HT-29 colon cancer cells (Ferguson, Zhu, & Harris, 2005). The upper crust extract of Louise whole wheat bread exhibited the highest inhibition of HT-29 cancer cells by 26.82% at 9.0 mg BE/mL, and 46.67% at 45.0 mg BE/mL at 96 hours of treatment **(Figure 4.1)**, while the upper crust extracts of Macon whole wheat bread inhibited HT-29 cancer cell by 23.53% at 9.0 mg

BE/mL, and 55.93% at 45.0 mg BE/mL at 96 hours of treatment **(Figure 4.2)**. The extracts of Louise and Macon whole wheat upper crust exhibited stronger inhibitory effects against HT-29 cancer cells than their crumb and dough at all three tested concentration, especially at the concentration of 45.0 mg BE/mL **(Figure 4.1 and 4.2)**.

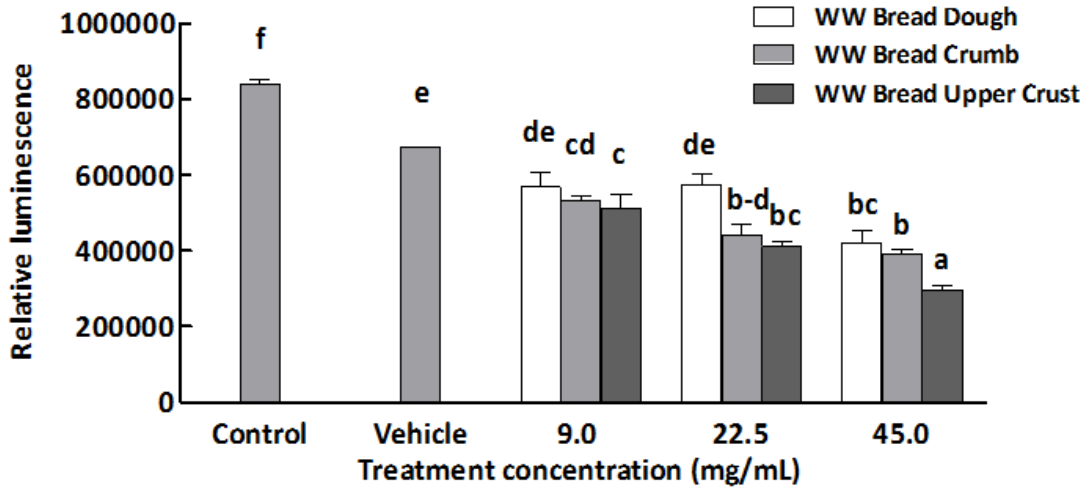

Figure 4.2. Antiproliferative effects of extracts from dough, crumb, and crust made from Macon (hard white wheat cultivar) whole wheat flour, in cultured HT-29 human colon cancer cells.
HT-29 cells (2.5 x 10^3 cells/well) were incubated overnight prior to treatments. Control, Culture medium only; Vehicle, Culture medium with 1% DMSO. Culture media was then removed and treatment media containing the extracts of dough, crumb, and crust were then added at the indicated concentrations and incubated for 96 hrs. Relative luminescence is proportional to the number of viable cells. Values were based on triplicate tests, with mean ± SD shown (n = 3). The statistical significance of samples is indicated using letters and results labeled by the same letter are not significantly different ($P > 0.05$).

Together, these data demonstrated that baking might enhance antiproliferative activities in bread, and increased thermal treatment may result in a greater availability of wheat antiproliferative components. These results are in agreement with the recent study with soft winter wheat flour by Lv et al. (2012). The authors observed reduced growth of HT-29 colon cancer cells at a concentration of 50 mg flour equivalent/mL after 48 hours

treatment. In addition, antiproliferative activity against HT-29 positively was somehow correlated with total insoluble bound phenolic acids (r = 0.515, $P < 0.05$) and total soluble phenolic acids (r = 0.641, $P < 0.001$). There were low positive correlations with the other two phytochemical components, total carotenoids (r =0.29, $P < 0.05$) and total tocopherols (r =0.23, $P < 0.05$) investigated in the present study (**Table.4.4**).

Table 4.4. Correlations between phytochemicals and antiproliferative activity [a]

	Lutein	Zeax	TC	α-Toco	δ-Toco	TT	TIPA	TSPA
Zeax	0.99***							
Total Caros	1.00***	1.00***						
α-Toco	0.99***	0.99***	0.99***					
δ-Toco	0.96***	0.96***	0.96**	0.95***				
Total Tocos	0.99***	0.99***	0.99***	1.00***	0.958***			
TIPA	0.92***	0.92***	0.92**	0.90***	0.968***	0.903*		
TSPA	0.15	0.15	0.15	0.10	0.371	0.117	0.451*	
HT-29	0.29	0.30	0.29	0.22	0.387	0.225	0.515*	0.641*

[a] Data represents Pearson Correlation Coefficient R. Zeax – Zeaxanthin; α-Toco - α-tocopherol; δ-Toco - δ-tocopherol; TC - total carotenoids; TT - Total tocopherols; TIPA - Total insoluble phenolic acids; TSPA - Total soluble phenolic acids; HT29, antiproliferation test against HT-29 colon cancer cells; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

4.5 Conclusions

In summary, ferulic acid was the most abundant phenolic acid in the dough, crumb and upper crust made with refined and whole-wheat flour in two wheat cultivars (Macon and Louise). Significantly higher amounts (around ten-fold) of phenolic acids were extracted from whole wheat as compared to refined wheat fractions. The quantity of phenolic acids in the upper crust fraction was higher than in the dough and crumb fractions, suggesting that total phenolic acids content, especially ferulic and *p*-coumaric acids were not lowered during baking. Lutein and α-tocopherol were the predominant carotenoid and tocopherol, respectively, extracted from all fractions. Overall, the contents of both carotenoids and tocopherols were at least three-fold higher in whole wheat fractions as compared to their counterparts made with refined wheat fraction. The dough had the highest levels of tocopherols and carotenoids compared to crumb and upper crust of bread, suggesting that possible degradation of tocopherols and carotenoids during baking. Antiproliferative activity against HT-29 was somewhat positively correlated with total insoluble phenolic acids and total soluble conjugated phenolic acids, but had lower positive correlations with other phytochemical components such as total carotenoids and total tocopherols. The results presented in this study will be of significant interest to bread manufacturers and nutrition professionals.

Summary and Future Research

The goal of this research was to promote the use of selected soft wheat cultivars to improve human health while enhancing food and agriculture economy. The current investigation discovered that soft wheat samples varied in their chemical composition, and antioxidant and antiproliferative properties. The genotypes and growing environments may as well alter biological properties of soft wheat bran samples. Furthermore, our results indicated that baking may not decrease total phenolic acids content in upper crust fraction of bread, but reduced the concentrations of carotenoids and tocopherols of breads.

Future research of soft wheat should include a more detailed investigation about the bioactive components. Bioaccessibility, bioavailability, and metabolic effect of bioactive components in *vivo* will be carried out to better understand soft wheat's health-beneficial effects. In addition, the bioactivity of wheat-based product is of significant interest and might be conducted in our future plan.

References

AACC. Methods 10-10B and 46-30. In Approved Methods of the American Association of Cereal Chemists, 10th Ed. The Association: St. Paul, MN, 2000.

Abdel-Aal, E. S. M., & Rabalski, I. (2013). Effect of baking on free and bound phenolic acids in whole grain bakery products. *Journal of Cereal Science, 57*, 312-318.

Adom, K. K., Sorrells, M. E., & Liu, R. H. (2005). Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. *Journal of Agricultural and Food Chemistry, 53*, 2297-2306.

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

Adom, K. F., Sorrells, M. E., & Liu, R. H. (2003). Phytochemical profiles and antioxidant activity of wheat varieties. *Journal of Agricultural and Food Chemistry, 51*, 7825-7834.

Alabaster, O., Tang, Z. C., Frost, A., & Shivapurkar, N. (1993). Potential synergism between wheat bran and psyllium: enhanced inhibition of colon cancer. *Cancer Letters, 75*, 53–58.

Albanes, D., Malila, N., Taylor, P., Kuttunen, J., Virtamo, J., Edwards, B., Rautalahti, M., Hartman, A., Barrett, M., & Pietinen, P. (2000). Effect of supplemental α-tocopherol and β-carotene on colorectal cancer: results from a controlled trial (Finland). *Cancer Causes and Control, 11*, 197-205.

Amarowicz, R., & Maramać, M. (2002). Antioxidant activity of wheat caryopses and embryo extracts. *Journal of Food Lipids, 9*, 201-210.

Anderson, J. W. (2004). Whole grains and coronary heart disease: the whole kernel of truth. *Proceedings of the Nutrition Society, 62*, 135-142.

Anson, N. M., Berg, R., Havenaar, R., Bast, A., & Haenen, G. R. M. M. (2009a). Bioavailability of ferulic acid is determined by its bioaccessibility. *Journal of Cereal Science, 49*, 296-300.

Anson, N. M., Selinheimo, E., Havenaar, R., Aura, A. M., Mattila, I., Lehtinen, P., Bast, A., Poutanen, K., & Haenen, G. R. M. M. (2009b). Bioprocessing of wheat

bran improves in vitro bioaccessibility and colonic metabolism of phenolic compounds. *Journal of Agricultural and Food Chemistry, 57,* 6148-6155.

Astorg, P., Boutron-Ruoult, M.C., Andrieux, C., Astorg, P., Blachier, F., Blottiere, H., Bonithon-Kopp, C., Boutron-Ruault, M.C., Cassand, P., Chaumontet, C., Cherbut, C., Clavel-Chapelon, F., Corpet, D., Duee, P.H., Gerber, M., Meflah, K., Menanteau, J., & Siess, M. H. (2002). Dietary fibers and colorectal cancer. Experimental studies, epidemiology, mechanisms. *Gastroenterologie Clinique Et Biologique, 26,* 893-912.

Atanackovic, M., Aleksandar P., Jovic, S., Gojkovic-Bukarica, L., Bursac, M., & Cvejic, J. (2012). Influence of winemaking techniques on the resveratrol content, total phenolic content and antioxidant potential of red wines. *Food Chemistry, 131*, 518-531.

Bardarinath, A. V., Rao, K. M., Chetty, C. M. S., Ramkanth, V., Rajan, T. V. S., Gnanaprakash, K. (2010). A review on in-vitro antioxidant methods: comparisons, correlations and considerations. *International Journal of PharmTech Research, 2,* 1276-1285.

Barbaste, M., Berke, B., Dumas, M., Soulet, S., Delaunay, J., Castagnino, C., Arnaudinaud, V., & Vercauteren, J. (2002). Dietary antioxidants, peroxidation and cardiovascular risks. *Journal of Nutrition Health and Aging, 6,* 209-223.

Barbolt, T.A., & Abraham, R. (1978). The effect of bran on dimethylhydrazine induced colon carcinogenesis in the rat. *Proceedings of the Society of Experimental Biology and Medicine, 157,* 656–659.

Barbolt, T.A., & Abraham, R. (1980). Dose response, sex difference and the effect of bran in dimethylhydrazine induced intestinal tumourigenesis in rats. *Toxicology and Applied Pharmacology, 55,* 417–422.

Baublis, A., Decker, E. A., & Clydesdale, F. M. (2000). Antioxidant effect of aqueous extracts from wheat based read-to-eat breakfast cereals. *Food Chemistry, 68,* 1-6.

Beta, T., Nam, S., Dexter, J. E., & Sapirstein, H. D. (2005). Phenolic content and antioxidant activity of pearled wheat and roller-milled fractions. *Cereal Chemistry, 82,* 390-393.

Bock, M. A. 2000. Minor constituents of cereals. Page 479-504 in: Handbook of Cereal Science and Technology, 2nd ed. K. Kulp and J. G. J. Ponte, Eds. Marcel Dekker, New York.

Borrelli, G. M., Troccoli, A., Di Fonzo, N., & Fares, C. (1999). Durum wheat lipoxygenase activity and other quality parameters that affect pasta color. *Cereal Chemistry, 76,* 335-340.

Briggle, L. W., Curtis, B. C. Wheat worldwide. In: Wheat and wheat improvement; Heyne, E.G. Eds. 1994; pp 1-31.

Brown, J., Byers, T., Thompson, K., Eldridge, B., Doyle, C., & Williams, A.M. (2001). Nutrition during and after cancer treatment: A guide for informed choices by cancer survivors. *CA: A Cancer Journal for Clinicians, 51,* 153−181.

Buri, R. C., Von Reding, W., & Gavin, M. H. (2004). Description and characterization of wheat aleurone. *Cereal Foods World, 49,* 274-278.

Carter, J.W., Madl, R., & Padula, F. (2006). Wheat antioxidants suppress intestinal tumor activity in Min mice. *Nutrition Research, 26,* 33-38.

Cheng, Z. H., Moore, J., & Yu, L. L. (2006). High-throughput relative DPPH radical scavenging capacity assay. *Journal of Agricultural and Food Chemistry, 54,* 7429-7436.

Cheng, Z., Zhou, H., Yin, J.-J., & Yu, L. (2007). Electron spin resonance estimation of hydroxyl radical scavenging capacity for lipophilic antioxidants. *Journal of Agricultural and Food Chemistry, 55,* 3325-3333.

Cheng, Z., Zhou, H., Luther, M., Yin, J., & Yu, L. (2008). Effects of wheat antioxidants on oxygen diffusion-concentration products in liposomes and mRNA levels of HMG-CoA reductase and cholesterol 7α-hydroxylase in primary rat hepatocytes. *Journal of Agricultural and Food Chemistry, 56,* 5033-5042.

Chung, O. K., & Ohm, J. 2000. Cereal lipids. Pages 417-477 in: Handbook of Cereal Science and Technology, 2nd ed. K. Kulp and J. G. J. Ponte, Eds. Marcel Dekker, New York.

Crosby, G. (2005). Lignans in food and nutrition. *Food Technology, 59,* 32-36.

Curtis, B.C. Wheat in the world. In: Bread wheat improvement and production; Curtis, B. C.; Rajaram, S.; Macpherson, H.G. Eds. Plant production and protection series. FAO (Rome, Italy), 2002; pp 1-19.

de Munter, J. S., Hu, F., Spiegelman, D., Franz, M., & van Dam, R.M. (2007). Wholegrain, bran and germ intake and risk of type 2 diabetes: a prospective cohort study and systematic review. *PLoS Medicine*, *4*, 1389–1395.

Diplock, A. T., Charleux, J. L., Crozier-Willi, G., Kok, F. J., Rice-Evans, C., Roberfroid, M., Stahl, W., & Vina-Ribes, J. (1998). Functional food science and defense against reactive oxidative species. *British Journal of Nutrition, 80*, 77-112.

FAO. 2010. Cereal Supply and Demand Brief. In Food and Agriculture Organization of the United States.

Farvin, K. H. S., & Jacobsen, C. (2013). Phenolic compounds and antioxidant activities of selected species of seaweeds from Danish coast. *Food Chemistry, 138*, 1670-1681.

Feldman, M., & Kislev, M. E. (2007). Domestication of emmer wheat and evolution of free-threshing tetraploid wheat in "A Century of Wheat Research-From Wild Emmer Discovery to Genome Analysis". *Israel Journal of Plant Sciences*, *55*, 207-221.

Ferguson, L. R., Zhu, S. T., & Harris, P. J. (2005). Antioxidant and antigenotoxic effects of plant cell wall hydroxycinnamic acids in cultured HT-29 cells. *Molecular Nutrition & Food Research, 49*, 585-593.

Finley, J. W., Kong, A., Hintze, K. J., Jeffery, E. H., Ji, L. L., & Lei, X. G. (2011). Antioxidants in Foods: State of the science important to the food industry. *Journal of Agricultural and Food Chemistry*, 59, 6837-6846.

Fridovich, I. (1998). Oxygen toxicity: A radical explanation. *Journal of Experimental Biology*, *201*, 1203-1209.

Fritsche, K. L., & Johnston, P. V. (1998). Rapid autoxidation of fish oil in diets without added antioxidants. *Journal of Nutrition, 118*, 425-426.

Fuchs, C. S., Giovannucci, E. L., & Colditz, G. A. (1999). Dietary fiber and the risk of colorectal cancer and adenoma in women. *New England Journal of Medicine*, *340*, 169-176.

Fulcher, R, G., & Duke, T, K. Whole-grain structure and organization: Implications for nutritionists and processors. In Whole-grain foods in health and disease, Marquart, L; Slavin, L; Fulcher, R, G., Eds.; American Association of Cereal Chemists: St. Paul, Minnesota, 2002; pp 9-45.

Fung, T. (2002). Whole-grain intake and the risk of type 2 diabetes: a prospective study in men. *American Journal of Clinical Nutrition, 76*, 535–540.

Giron, M. V., Ruiz-Jimenez, J., & Luque de Castro, M. D. (2009). Dependence of fatty-acids composition of edible oils on their enrichment in olive phenols. *Journal of Agricultural and Food Chemistry*, *57*, 2797–2802.

Graf, E., & Eaton, J. W. (1990). Antioxidant functions of phytic acid. *Free Radical Biology and Medicine, 8,* 61-69.

Guntensperger, B., Hammerli-Meier, D. E., & Escher, F. E. (1998). Rosemary extract and precooking effects on lipid oxidation in heat sterilized meat. *Journal of Food Science*, *63*, 955–957.

Hakala, P., Lampi, A., Ollilainen, V., Werner, U., Murkovic, M., Wahala, K., Karkola, S., & Piironen, V. (2002). Steryl phenolic acid esters in cereals and their milling fractions. *Journal of Agricultural and Food Chemistry*, *50*, 5300-5307.

Halliwell, B., & Whiteman, M. (2004). Measuring reactive species and oxidative damage in vivo and in cell culture: How should you do it and what do the results mean? *British Journal of Pharmacology*, *142*, 231-255.

Hansen, H. B., Andreasen, M. F., Nielsen, M. M., Larsen, L. M., Knudsen, K. E. B., Meyer, A. S., Christensen, L. P., & Hansen, A. (2002). Changes in dietary fiber, phenolic acids and activity of endogenous enzymes during rye bread-making. *European Food Research and Technology, 214*, 33-42.

Heinonen, M., Ollilainen, V., Linkola, E., Varo, P., & Koivistoinen, P. (1989). Carotenoids and retinoids in Finnish foods: cereal and bakery products. *Cereal Chemistry, 66*, 270-273.

Hemery, Y. M., Anson, N. M., Havenaar, R., Haenen, G. R. M. M., Noort, M. W. J., & Rouau, X. (2012). Dry-fractionation of wheat bran increases the bioaccessibility of phenolic acids in breads made from processed bran fractions. *Food Research International*, *43*, 1429-1438.

Hidalgo, A., & Brandolini, A. (2010a). Tocols stability during bread, water biscuit and pasta processing from wheat flours. *Journal of Cereal Science*, *52*, 254-259.

Hidalgo, A., Brandolini, A., & Pompei, C. (2010b) Carotenoids evolution during pasta, bread and water biscuit preparation from wheat flours. *Food Chemistry, 121*, 746-751.

Holasova, M. (1997). Distribution of tocopherols and tocotrienols in the main products of wheat and rye milling. *Czech Journal of Food Sciences*, *15*, 343-350.

Hoseney, R. C., Wade, P., & Finley, J. W. (1988). Soft wheat products. In Y. Pomeranz. (Eds), Wheat: Chemistry and Technology (pp. 407-456). St. Paul, MN: American Association of Cereal Chemists.

Huang, D., Ou, B., Hampsh-Woodill, Flanagan, J. A., & Prior, R. L. (2001). 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. *Journal of Agricultural and Food Chemistry*, *50*, 4437-4444.

Humphries, J. M., & Khachik, F. (2003). Distribution of lutein, zeaxanthin, and related geometrical isomers in fruit, vegetables, wheat, and pasta products. *Journal of Agricultural and Food Chemistry, 51*, 1322-1327.

Izydorczyk, M. S., Symons, S. J., & Dexter, J. E. Fractionation of wheat and barley. In Whole-grain foods in health and disease, Marquart, L; Slavin, L; Fulcher, R, G., Eds.; American Association of Cereal Chemists: St. Paul, Minnesota, 2002; pp 47-82.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E., & Forman, D. (2011). Global cancer statistics. *CA: A Cancer Journal for Clinicians, 61*, 69-90.

Jenab, M., & Thompson, L. U. (1998). The influence of phytic acid in wheat bran on early biomarkers of colon carcinogenesis. *Carcinogenesis, 19*, 1087-1092.

Jensen, M. K., Koh-Banerjee, P., Hu, F. B., Franz, M., Sampson, L., Gronbaek, M., Rimm, E. B. (2004). Intakes of whole-grains, bran, and germ and the risk of coronary heart disease in men. *American Journal of Clinical Nutrition, 80*, 1492-1499.

Jones, J. M. (2006). Grain-based foods and health. *Cereal Food World, 51*, 108-113.

Kalantzakis, G., Blekas, G. (2006). Effect of Greek sage and summer savory extracts on vegetable oil thermal stability. *European Journal of Lipid Science and Technology, 108*, 842–847.

Kern, S. M., Bennett, R. N., Mellon, F. A., Kroon, P. A., & Garcia-Conesa, M. T. (2003). Absorption of hydroxycinnamates in humans after high-bran cereal consumption. *Journal of Agricultural and Food Chemistry, 51*, 6050-6055.

Khaw, K.T., Bingham, S., Welch, A., Luben, R., Wareham, N., & Oakes, S. (2001). Relation between plasma ascorbic acid and mortality in men and women in EPIC-Norfolk prospective study: a prospective population study. *EPIC, 357*, 657-663.

Kim, K., Tsao, R., Yang, R., & Cui, S. W. (2006). Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effects of hydrolysis conditions. *Food Chemistry, 95*, 466-473.

Koh-Banerjee, P., Franz, M., Sampson, L., Liu, S., Jacobs, D. R., & Spiegelman, D. (2004). Changes in wholegrain, bran and cereal fiber consumption in relation to 8 year weight gain among men. *American Journal of Clinical Nutrition, 80*, 1237–1245.

Kumar, S. (2011). Free radicals and antioxidants: human and food system. *Advanced in Applied Science Research, 2*, 129-135.

Leehardt, F., Lyan, B., Rock, E., Boussard, A., Potus, J., Chanliaud, E., & Remesy, C. (2006). Wheat lipoxygenase activity induces greater loss of carotenoids than vitamin E during breadmaking. *Journal of Agricultural and Food Chemistry, 54*, 1710-1715.

Li, W., Shan, F., Sun, S., Corke, H., & Beta, T. (2005). Free radical scavenging properties and phenolic content of Chinese black-grained wheat. *Journal of Agricultural and Food Chemistry, 53*, 8533-8536.

Li, L., Shewry, P., & Ward, J. L. (2008). Phenolic acids in wheat varieties in the HEALTHGRAIN diversity screen. *Journal of Agricultural and Food Chemistry, 56,* 9732-9739.

Liyana-Pathirana, C. M., Dexter, J., & Shahidi, F. (2006a). Antioxidant properties of wheat as affected by pearling. *Journal of Agricultural and Food Chemistry, 54,* 6177-6184.

Liyana-Pathirana, C. M., & Shahidi, F. (2006b). Importance of insoluble-bound phenolics to antioxidant properties of wheat. *Journal of Agricultural and Food Chemistry, 54,* 1256-1264.

Liyana-Pathirana, C. M., & Shahidi, F. (2006c). Antioxidant properties of commercial soft and hard wheats (*Triticum aestivum* L.) and their milling fractions. *Journal of the Science and Food Agriculture, 86,* 477-485.

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

Liyana-Pathirana, C. M., & Shahidi, F. (2008). The antioxidant potential of milling fractions from bread, wheat and durum. *Journal of Cereal Science, 45,* 238–247.

Lopez-Alarcon, C, & Denicola, A. (2013). Evaluating the antioxidant capacity of natural products: A review on chemical and cellular-based assays. *Analytica Chimica Acta, 763,* 1-10.

Luthria, D.L. (2006). Significance of sample preparation in developing analytical methodologies for accurate estimation of bioactive compounds in functional foods. *Journal of the Science and Food Agriculture, 86,* 2266-2272.

Lv, J., Yu, L., Lu, Y., Niu, Y., Liu, L., Costa, J., & Yu, L. (2012). Phytochemical compositions, and antioxidant properties, and antiproliferative activities of wheat flour. *Food Chemistry, 135,* 325-331.

Lv, J., Lu, Y., Niu, Y., Whent, M., Ramadan, M. F., Costa, J., Luthria, D., & Yu, L. (2013). Effect of genotype, environment, and their interaction on phytochemical compositions and antioxidant properties of soft winter wheat flour. *Food Chemistry, 138,* 454-462.

Magalhaes, L. M., Segundo, M. A., Reis, S., & Lima, J. L. F. C. (2008). Methodological aspects about in vitro evaluation of antioxidant properties. *Analytica Chimica Acta*, *613*, 1-19.

Martinez-Tome, M., Murcia, M. A., Frega, N., Ruggieri, S., Jimenez, M., Roses, F., & Parras, R. (2004). Evaluation of antioxidant capacity of cereal brans. *Journal of Agricultural and Food Chemistry*, *52*, 4690-4699.

Mateo Anson, N., Van den Berg, R., Havenaar, R., Bast, A., & Haenen, G.R. (2008). Ferulic acid from aleurone determines the antioxidant potency of wheat grain (Triticum aestivum L.). *Journal of Agricultural and Food Chemistry*, *56*, 5589–5594.

Mattila, P., Pihlava, J., & Hellström, J. (2005). Contents of phenolic acids, alkyl-and alkenylresorcinols, and avenanthramides in commercial grain products. *Journal of Agricultural and Food Chemistry*, *53*, 8290-8295.

Mellen, B. P., Walsh, T. F., & Herrington, D. M. (2009). Whole-grains intake and cardiovascular disease: a meta-analysis. *Nutrition, Metabolism and Cardiovascular Diseases*, *18*, 283–290.

Michalska, A., Amigo-Benavent, M., Zielinski, H., & Del Castillo, M. D. (2008). Effect of bread making on the formation of Maillard reaction products contributing to the overall antioxidant activity of rye bread. *Journal of Cereal Science*, *48*, 123-132.

Moore, J., Hao, Z., Zhou, K., Luther, M., Costa, J., & Yu, L. (2005). Carotenoid, tocopherol, phenolic acid, and antioxidant properties of Maryland-grown soft wheat. *Journal of Agricultural and Food Chemistry*, *53*, 6649-6657.

Moore, J., Liu, J. G., Zhou, K., & Yu, L. (2006a). Effects of genotype and environment on the antioxidant properties of hard winter wheat bran. *Journal of Agricultural and Food Chemistry*, *54*, 5313-5322.

Moore, J., Luther, M., Cheng, Z., & Yu, L. (2009). Effects of baking conditions, dough fermentation and bran particles size on antioxidants properties of whole-wheat pizza crusts. *Journal of Agricultural and Food Chemistry*, *57*, 832-839.

Moore, J., Yin, J. J., & Yu, L. (2006b). Novel fluorometric assay for hydroxyl radical scavenging capacity (HOSC) estimation. *Journal of Agricultural and Food Chemistry, 54*, 617-626.

Morris, C. F., Campbell, K. G., & King, G. E. (2005). Kernel texture differences among US soft wheat cultivars. *Journal of the Science of Food and Agriculture, 85*, 1959-1965.

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

Noort, M. W. J., Haaster, D. V., Hemery, Y., Schols, H. A., & Hamer, R. J. (2010). The effect of particle size of wheat bran fractions on bread quality- Evidence for fibre-protein interactions. *Journal of Cereal Science, 52*, 59-64.

Nystrom, L., Paasonen, A., Lampi, A. M., & Piironen, V. (2007). Total plant sterols, steryl ferulates and steryl glycosides in milling fractions of wheat and rye. *Journal of Cereal Science, 45,* 106-115.

Panfili, G., Alessandra, F., & Irano, M. (2003). Normal phase high-performance liquid chromatography method for the determination of tocopherols and tocotrienols in cereals. *Journal of Agricultural and Food Chemistry, 51*, 3940-3944.

Park, Y., Hunter, D. J., & Spiegelman, D. (2005). Dietary fiber intake and risk of colorectal cancer - A pooled analysis of prospective cohort studies. *Journal of the American Medical Association, 294*, 2849-2857.

Parker, M. L., Ng, A., Waldron, K. W. (2005). The phenolic acid and polysaccharide composition of cell walls bran layers of mature wheat (Triticum aestivum L. cv. Avalon) grains. *Journal of Agricultural and Food Chemistry, 85*, 2539-2547.

Peña, R. J. Wheat for bread and other foods. In: Bread wheat improvement and production; Curtis, B. C.; Rajaram, S.; Macpherson, H.G. Eds. Plant production and protection series. FAO (Rome, Italy), 2002.

Piironen, V., Lampi, A. M., Ekholm, P., Salmenkallio-Marttila, M., & Liukkonen, K. H. (2009). Micronutrients and phytochemicals in wheat grain. Pages: 179-222 in:

Wheat, Chemistry and Technology, 4th edition. Khan, K. and Shewry, P. R., eds. AACC International: St. Paul, MN, USA.

Piironen, V., Syvaoja, E., Varo, P., Salminen, K., & Koivistoinen, P. (1986). Tocopherols and tocotrienols in cereal products from Finland. *Cereal Chemistry*, *63*, 78-81.

Piironen, V., Toivo, J., Lampi, A. M. (2002). Plant sterols in cereals and cereal products. *Cereal Chemistry*, *79*, 148-154.

Pomeranz, Y. Chemical composition of kernel structures. (1988). Pages 97-185 in Wheat: Chemistry and Technology, 3rd ed., Vol.1 Y. Pomeranz, Ed. American Association of Cereal Chemistry, St. Paul, MN.

Prior, R. L., Wu, X., & Schaich, K. (2005). Standardized methods for determination of antioxidant capacity and phenolics in foods and dietary supplements. *Journal of Agricultural and Food Chemistry*, *53*, 4290-4302.

Ranhotra, G. S., Gelroth, J. A., Langemeier, J., & Rodgers, D. E. (1995). Stability and contribution of beta carotene added to whole wheat bread and crackers. *Cereal Chemistry*, 72, 139-141.

Reboul, E., Richelle, M., Perrot, E., Desmoulins-Malezet, C., Pirisi, V., & Borel, P. (2006). Bioaccessibility of carotenoids and vitamin E from their main dietary sources. *Journal of Agricultural and Food Chemistry, 54*, 8749-8755.

Reddy, B. S., Hirose, Y., Cohen, L. A., Simi, B., Cooma, I., & Rao, C. (2000). Preventive potential of wheat bran fractions against experimental colon carcinogenesis: implications for human colon cancer prevention. *Cancer Research, 60*, 4792-4797.

Reddy, B. S., & Mori, H. (1981). Effect of dietary wheat bran and dehydrated citrus fiber on 3, 2-dimethyl-4-aminobiphenyl induced intestinal carcinogenesis in F344 rats. *Carcinogenesis*, *2*, 21–25.

Rodgers, D. E., Malouf, R. B., Langemeier, J., Gelroth, J. A., & Ranhotra, G. S. (1993). Stabililty and nutrient contribution of beta-carotene added to selected bakery products. *Cereal Chemistry*, *70*, 558-561.

Saura-Calixto, F. (2011). Dietary fiber as a carrier of dietary antioxidants: an essential physiological function. *Journal of Agricultural and Food Chemistry*, *59*, 43-49.

Sayhoun, N. R., Jacques, P. F., Zhang, X. L., Juan, W., & McKeown, N. M. (2006). Whole grain is inversely associated with the metabolic syndrome and mortality in older adults. *American Journal of Clinical Nutrition, 83,* 124–131.

Schatzkin, A., Mouw, T., Park, Y., Subar, A.F., Kipnis, V., Hollenbeck, A., Leitzmann, M. F., & Thompson, F. E. (2007). Dietary fibre and wholegrain consumption in relation to colorectal cancer in the NIH AARP diet and health study. *American Journal of Clinical Nutrition, 85,* 1353–1360.

Seal, C. J. (2006). Whole-grains and CVD risk. *Proceedings of the Nutrition Society, 65,* 24–34.

Shahidi, F. In: Natural Antioxidant: Chemistry, Health Effects and Applications. Shahidi, F. Eds. AOCS Press. Champaign. IL, 1997; pp 1-11.

Shahidi. F., & Zhong, Y. (2010). Novel antioxidants in food quality preservation and health promotion. *European Journal of Lipid Science and Technology, 112,* 930-940.

Shewry, P. R. (2007). Improving the protein content and composition of cereal grain. *Journal of Cereal Science, 46,* 239–250.

Shewry, P. R. (2009). The health-grain program opens new opportunities for improving wheat for nutrition and health. *Nutrition Bulletin, 34,* 225–231.

Shewry, P., Piironen, V., Lampi, A., Edelmann, M., Kariluoto, S., & Nurmi, T. (2010). The healthgrain wheat diversity screen: effects of genotype and environment on phytochemicals and dietary fiber components. *Journal of Agricultural and Food Chemistry, 58,* 9291-9298.

Singleton, V.L., Orthofer, R., & Lamuela-Raventos, R.M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymology, 299,* 152-178.

Slavin, J. (2004). Whole grains and human health. *Nutrition Research Reviews, 17,* 99-110.

Slavin, J. L., Jacobs, D., & Marquart, L. (2000). Grain processing and nutrition. *Critical Reviews in Food Science and Nutrition, 40,* 309-326.

Slavin, M., Kenworthy, W., & Yu, L. (2009). Antioxidant properties, phytochemical composition, and antiproliferative activity of Maryland-grown soybeans with colored seed coats. *Journal of Agricultural and Food Chemistry*, *57*, 11174-11185.

Slavin, J., Marquart, L., & Jacobs, D. (2000). Consumption of whole-grain foods and decreased risk of cancer: proposed mechanisms. *Cereal Food World*, *45*, 54-58.

Soh, N. (2006). Recent advances in fluorescent probes for the detection of reactive oxygen species. *Analytical and Bioanalytical Chemistry*, *386*, 532-543.

Stahl, W., Van den Berg, H., Arthur, J., Bast, A., Daninty, J., Faulks, R. M. et al. (2002). Bioavailability and metabolism. *Molecular Aspects of Medicine, 23*, 39-100.

Stone, W. L., Papas, A. (2003). Tocopherols, tocotrienols and Vitamin E. Pages 53-72 in : Lipids for functional foods and nutraceuticals. F. D. Gunstone, Ed. The oily Pres, Bridgewater, England.

Temple, N. J. (2000). Antioxidants and disease: More questions than answers. *Nutrition Research, 20*, 449-459.

Trowell, H. (1972). Ischemic heart disease and dietary fiber. *American Journal of Clinical Nutrition, 25*, 926-932.

Truswell, A. S. (2002). Cereal grains and coronary heart disease. *European Journal of Clinical Nutrition, 56*, 1-14.

USDA. (2005). Will 2005 the year of the whole-grain? Amber Waves, 3, 12-17. Available online:http://www.ers.usda.gov/AmberWaves/June05/Features?Will2005WholeGrain.htm.

USDA. (2006). Agricultural Baseline Projections to 2015, February, OCE-2006. Accessed November 2006.http://www.ers.usda.gov/publications/oce061/oce20061.pdf

USDA. (2012). http://www.ers.usda.gov/topics/crops/wheat.aspx. Accessed, 17.11.2012.

USDA. Grain: World Market and Trade. (2013a). URL http://fas.usda.gov/grain/current/#wheat. Accessed 10.05.2013

USDA. Wheat Background. (2013b). URL http://ers.usda.gov/topics/crops/wheat/background. aspx#. Accessed. 25.01.2013

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *International Journal of Biochemistry and Cell Biology*, *39*, 44-84.

Velioglu, Y. S., Mazza, G., Gao, L., & Oomah, B. D. (1998). Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *Journal of Agricultural and Food Chemistry,* 46, 4113-4117.

Verma, B., Hucl, P., & Chibbar, R. N. (2009). Phenolic acid composition and antioxidant capacity of acid and alkali hydrolysed wheat bran fractions. *Food Chemistry*, *116*, 947-954.

Veronica, S. M., Feliciano, P. C., Carlos, J., & Mara, D. L. C. (2011). Quality and stability of edible oils enriched with hydrophilic antioxidants from the olive tree: the role of enrichment extracts and lipid composition. *Journal of Agricultural and Food Chemistry*, *59*, 11432–11441.

Vetter, J. L. (1988). Commercially available fiber ingredients and bulking agents. *American Institute of Baking Research Department Technical Bulletin*, *10*, 5-8.

Virgili, F. & Marino, M. (2008). Regulation of cellular signals from nutritional molecules: A specific role for phytochemicals, beyond antioxidant activity. *Free Radical Biology and Medicine*, *45,* 1205–1216.

Wang, C. Y., Wang, S. Y., Yin, J. J., Parry, J., & Yu, L. L. (2007). Enhancing antioxidant, antiproliferation, and free radical scavenging activities in strawberries with essential oils. *Journal of Agricultural and Food Chemistry, 55*, 6527- 6532.

Wang, T., He, F., & Chen, G. (2014). Improving bioaccessibility and bioavailability of phenolic compounds in cereal grains through processing technologies: A concise review. *Journal of Functional Foods*, jff.2014.01.033

Ward, J. L., Poutanen, K., Gebruers, K., Piironen, V., Lampi, A. M., Nyström, L., Andersson, A. A. M., Åman, P., Boros, D., Rakszegie, M., Bedõ, Z., & Shewry, P. R. (2008). The HEALTHGRAIN cereal diversity screen: concept,

results and prospects. *Journal of Agricultural and Food Chemistry*, *56*, 9699-9709.

Wardman, P. (2008). Use of Dichlorofluoescein assay to measure “reactive oxygen species”. *Radiation Research, Letter to the Editor*, *170*, 406-407.

Whent, M., Huang, H., Xie, Z., Lutterodt, H., Yu, L., Fuerst, E., Morris, C., Yu, L., & Luthria, D. (2012). Phytochemical composition, anti-inflammatory, and antiproliferative activity of whole wheat flour. *Journal of Agricultural and Food Chemistry*, *60*, 2129-2135.

Willcox, J. K., Ash, S. L., & Catignani, G. L. (2004). Antioxidants and prevention of chronic disease. *Critical Reviews in Food Science and Nutrition*, *44*, 275-295.

William, R. M., O’Brien, L., Eagles, H. A., Solah, V. A., & Jayasena, V. (2008). The influences of genotype, enviroment, and genotype × enviroment interaction on wheat quality. *Australian Journal of Agricultural Research*, *59*, 95-111.

Wolfe, K. L., & Liu, R. H. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidants, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry*, *55*, 8896-8907.

Yeng, Y., McDonald, C. E., & Vick, B. A. (1988). C-glycosylflavones from hard red spring wheat bran. *Cereal Chemistry*, *65*, 452-456.

Yu, L., Haley, S., Perret, J., Harris, M., Wilson, J., & Qian, M. (2002). Free radical scavenging properties of wheat extracts. *Journal of Agricultural and Food Chemistry*, *50*, 1619-1624.

Yu, L., Perret, J., Harris, M., Wilson, J., & Haley, S. (2003). Antioxidant properties of bran extracts from “Akron” wheat grown at different locations. *Journal of Agricultural and Food Chemistry*, *51*, 1566-1570.

Yu, L., & Zhou, K. (2004). Antioxidant properties of bran extracts from 'Platte' wheat grown at different locations. *Food Chemistry*, *90*, 311-316.

Yu, L., Zhou, K., & Parry, J. W. (2005). Inhibitory effects of wheat bran extracts on human LDL oxidation and free radicals. *LWT – Food Science and Technology*, *38*, 463-470.

Yu, L. L., Haley, S., Perret, J., & Harris, M. (2002). Antioxidant properties of hard winter wheat extracts. *Food Chemistry*, *78*, 457-461.

Zile, M. H., Welsch, C. W., Welsch, M. A. (1998). Effect of wheat bran fiber on the development of mammary tumors in female intact and overiectomized rats treated with 7, 12-dimethylbenz(a)anthracene and in mice with spontaneously developing mammary tumors. *International Journal of Cancer*, *75*, 439-443.

Zhou, K., Su, L., & Yu, L. (2004a). Phytochemicals and antioxidant properties in wheat bran. *Journal of Agricultural and Food Chemistry, 52,* 6108-6114.

Zhou, K., Laux, J. J., & Yu, L. (2004b). Comparison of Swiss red wheat grain, and fractions for their antioxidant properties. *Journal of Agricultural and Food Chemistry*, *52*, 1118-1123.

Zhou, K., Yin, J. J., & Yu, L. (2005). Phenolic acid, tocopherol and carotenoid compositions, and antioxidant functions of hard red winter wheat bran. *Journal of Agricultural and Food Chemistry, 53,* 3916-3922.

Zhou, K., & Yu, L. (2004c). Antioxidant properties of bran extracts from Trego wheat grown at different locations. *Journal of Agricultural and Food Chemistry*, *52,* 1112-1117.

Zielinski, H., Kozlowska, H., & Lewczuk, B. (2001). Bioactive compounds in the cereal grains before and after hydrothermal processing. *Innovative Food Science and Emerging Technologies*, *2*, 159-169.