ABSTRACT

Title of Dissertation: PHYTOCHEMICAL PROFILES AND ANTIOXIDANT PROPERTIES OF WHEAT

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The present study examined and compared wheat grain, bran, aleurone, and micronized aleurone for their antioxidant properties and phytochemical profiles. The results showed that wheat antioxidants were concentrated in bran fraction, particularly in the aleurone layer of wheat bran. Micronized aleurone demonstrated the highest antioxidant activities (scavenging activities against $ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC, and chelating activity), and the highest total phenolics and phenolic acids concentration, suggesting that post-harvest treatment may affect the availability of wheat antioxidants. The present +study also investigated bran samples of seven wheat varieties from four different countries for their antioxidant properties and phytochemical profiles. The bran samples exerted enormous differences in concentrations among their phenolic acids, tocopherols and carotenoids. Their antioxidant activities were also notably different from 13% (scavenging capacity against $O_2^{\bullet-}$) to 99% (ORAC). These data suggest that wheat varieties and growing conditions might alter wheat antioxidant properties and phytochemical compositions. In addition, eight selected Maryland grown soft wheat varieties or experimental lines were examined for potential beneficial components and antioxidant properties. These soft wheat varieties significantly differed in their $ABTS^{\bullet+}$ scavenging activity and chelating activity. Significant amount of phenolic acids have

been demonstrated in all soft wheat grain. But their phenolic acid content markedly differed among different varieties which mainly (89% - 94%) existed as the insoluble bound form with ferulic acid as the predominant phenolic acid. These data suggest the possibility to produce soft wheat varieties rich in selected health beneficial factors for optimum human nutrition through breeding programs. Finally, five phenolic acids commonly present in wheat grain and fractions were examined and compared for their radical scavenging properties and chelating capacities using spectrophotometric and electron spin resonance measurements. These phenolic acids differed in their properties to react with and quench $HO^{\bullet}$, $O_2^{\bullet-}$, $ABTS^{\bullet+}$, and $DPPH^{\bullet}$, as well as their capacities to form chelating complexes with transition metals. Strong structure-activity relationships were observed in the present study. Both substitutes on the phenyl ring and the conjugated carbon skeleton may influence the antioxidant properties of these phenolic acids.

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8-OHdG	8-hydroxy-2-deoxyguanosine
AAPH	2,2'-azobis (2-amino-propane) dihydrochloride
ABTS	2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt
ACF	Aberrant crypt foci
AMD	Advanced macular degeneration
AOM	Azoyxmehtane
BHA	Butylated hydroxyanisole
BHT	Butylatyed hydroxytoluene
BMPO	5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide
CHD	Coronary heart disease
CID	Collision induced dissociation
CLA	Conjugated linoleic acid
COPD	Chronic obstructive pulmonary disease
DPPH	2,2-diphenyl-1picrylhydrazyl
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Disodium ethylenediaminetetracetate
EPICN	European Prospective Investigation into Cancer and Nutrition
ESI	Electrospray ionization
ESR	Electron spin resonance
ET	Electron transfer
F-C reagent	Folin-Ciocalteu reagent
FL	Fluorescein
FOX	Ferrous oxidation-xylenol orange
FRAP	Ferric reducing/antioxidant power
GC	Gas chromatography
GE	Gallic acid equivalent
GPX	Glutathione peroxidase
HAT	Hydrogen atom transfer

$HO^{\bullet}$	Hydroxyl radical
HPLC	High performance liquid chromatography
HPLC-DAD-	High performance liquid chromatography-diode-array-detector-
ESI-MSMS	Electrospray ionization tandem mass spectrometry
HPX	Hypoxanthine
IQ	2-amino-3-methylimidazo[4,5- f]quinoline
LDL	Low density lipoprotein
MDA	Malondialdehyde
MRFIT	Multiple Risk Factor Intervention Trial
NBT	Nitro blue tetrazolium
NHANES II	National Health and Nutrition Examination Survey
$O_2^{\bullet-}$	Superoxide anion radical
ORAC	Oxygen radical absorbance capacity
OSI	Oxidative stability index
RDA	Recommended daily allowance
RMCD	Randomly methylated beta-cyclodextrin
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SRM	Selected reactant monitoring
TBA	Thiobarbituric acid
TBARS	TBA-reactive substance
TBHQ	Tert-butylhydroquinone
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic content
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
TSQ	N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide
XOD	Xanthine oxidase

INTRODUCTION

Recently, health benefits have become a critical marketing factor of food products primarily because of increased consumer awareness of the role of diet in health promotion and disease prevention. Consequently, antioxidants have received increasing attention due to their role of delaying or preventing oxidative stress resulting from the generation of reactive oxygen species (ROS) during metabolism. Previous studies showed that grain samples of selected hard winter wheat varieties had significant antioxidant activities and might serve as dietary sources of natural antioxidant. Wheat is the most widely grown crop in the world and continues to be the most important food ingredient for humans. US growers have suffered from the record low domestic price of wheat grain, and are looking for alternative approaches to enhance the market competitiveness. Characterization of wheat antioxidant properties and phytochemical profiles may lead to the improved utilization of US wheat in preparing food products rich in natural antioxidants for health promotion and disease prevention, while benefiting local agricultural economy. The specific objectives of this research were:

1. To examine and compare wheat grain, bran, aleurone, and micronized aleurone for their antioxidant properties and phytochemical profiles.
2. To evaluate bran samples of seven wheat varieties from four different countries for their antioxidant properties and phytochemical profiles.
3. To determine phenolic acid profiles and antioxidant properties of Maryland-grown soft wheat varieties.
4. To study antioxidant properties of wheat phenolic acids and the relationship between their antioxidant properties and chemical structures.

Chapter 1. LITERATURE REVIEW

Antioxidants and human health

Epidemiological studies have strongly indicated that the dietary antioxidants are highly associated with the reduced risks of chronic diseases such as heart disease, cancer, diabetes, neurological disease, immune disease, and eye diseases (*1-5*). The largest dietary intake study of antioxidants in relation to cardiovascular disease included 121,000 U.S. female nurses. The results demonstrated an inverse relationship of vitamin E and β-Carotene intake to coronary heart disease (CHD) & stroke following 8 years antioxidants intake questionnaire (*6-8*). The same results have also been suggested by other cohort studies conducted in various countries and districts (*9-11*). Coronary heart disease has been associated with reactive oxygen species (ROS) and oxidative damage to arterial endothelial cells and circulating lipids. Oxidized LDL plays a key role in the development of atherosclerosis (*1*). Oxidized LDL cholesterol is preferentially taken up by macrophages to create the foam cells characteristic of fatty streaks, which are precursors of atherosclerotic plaques. Antioxidants (particularly Vitamin E, which is carried primarily in the LDL cholesterol) help to maintain oxidant/antioxidants balance as well as inhibit the oxidation of LDL, therefore reducing the risk of cardiovascular disease (*4, 12*).

More evidence has been purported by blood-based cohort studies. In a 7 years follow up study, Gey et al (*13*) reported that a significant inverse association between human plasma vitamin E concentration and subsequent cancer mortality. Later, two cohort studies involving 36,265 and 12,866 subjects further supported the inverse association of plasma vitamin E and incidence of cancer (*14, 15*). A 12-year follow up

investigation of the NHANES II (National Health and Nutrition Examination Survey) cohort of US adults found men in the lowest serum ascorbate quartile to have a 62% higher risk of dying from cancer than men in the highest quartile (*16*). Similarly, the UK arm of the European Prospective Investigation into Cancer and nutrition (EPIC) found an inverse association between plasma ascorbic acid concentration and cancer mortality in men (a rise of 20 μmol/L in plasma ascorbic acid concentration was associated with a 21% reduction in cancer risk) (*17*). Antioxidants for cancer prevention still remained unclear to date, although several hypotheses have been proposed. The oxidative hypothesis of carcinogenesis asserts that carcinogens generate reactive oxygen species that damage RNA and DNA in cells, predisposing these cells to malignant changes. Antioxidants are proposed to prevent cell damage by neutralizing free radicals and oxidants, thus prevent subsequent development of cancer (*5*). Oxidation and generation of free radicals is an essential component of cell-mediated immunity. Antioxidants can improve immune response by controlling the production of free radicals in the cell. Supplementation with combination of vitamin E and C has been found to improve immune activities of neutrophils in compromised individuals (*18*). The generation of oxygen free radicals by activated inflammatory cells produces many of the pathophysiological changes associated with chronic obstructive pulmonary disease (COPD) such as asthma and bronchitis. Antioxidant nutrients have been suggested to play a role in the prevention and treatment of these conditions. Vitamin C is the major antioxidant present in the extracellular fluid lining of the lung, and intake in the general population has been inversely correlated with the incidence of asthma and bronchitis (*19, 20*). The eye disease case control study (*21*) reported an association between decreased

serum carotenoids and an increased risk of advanced macular degeneration (AMD), while increasing the intake of lutein and zeaxanthin from dietary sources significantly (58%) reduce the relative risk of AMD (*22*). In generally, antioxidants help to maintain oxidative-antioxidative balance in vivo primarily through one or more of the following pathways: scavenging free-radicals, chelating transition metals and quenching singlet oxygen (*23*). Thus, foods containing numerous nonessential compounds that possess antioxidant activity may help increase the antioxidant defense system of humans, a circumstance which, in turn, would benefit overall health.

Antioxidants and food quality & stability

Antioxidants also play an important role in preventing undesirable changes in flavor and nutritional quality of foods. The addition of 0.02% t- butylhydroquinone to fish oil can prevent the deterioration for at least 72 h and can significantly reduce the peroxide value (*24*). Some natural antioxidants, including vitamin E, soy protein isolates, cherry tissue, and rosemary extracts, were reported to be effective against lipid oxidation in meat products (*25, 26*), several food systems (*27*), bulk oils and oil-in-water emulsions (*28, 29*). During the storage of food, transition metals, light or other active sources may initiate the generation of free radicals starting free radical mediated lipid peroxidation (*30*). Free radicals also degrade proteins, vitamins and pigments, cross-link lipids and other macromolecules into non-nutritive polymers. This has been a problem of major concern for food quality and safety. Antioxidants can inhibit or retard lipid oxidation through chelating metal ions, scavenging peroxyl and alcoxyl radicals derived from unsaturated fatty acid oxidation, and quenching singlet oxygen (*31*). This results in interruption of continued lipid radical formation, therefore reduces auto-oxidation and an

increased shelf life of food products. It is for these reasons food manufacturers have used food-grade antioxidants to prevent quality deterioration of products and to maintain their nutritional value (*24, 32*). Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylatyed hydroxytoluene (BHT) have been used as antioxidants since the beginning of this century for this purpose.

Safety concerns of synthetic antioxidants

Synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylatyed hydroxytoluene (BHT) have been used as antioxidants since the beginning of this century. However, results of toxicological studies which link some synthetic antioxidants to cancer and other diseases have forced regulatory agencies to impose severe restrictions on their use in human foods (*33-36*). Thus, BHA is no longer allowed for food application in Japan and a number of other countries and TBHQ is banned in Canada, Japan and European countries (*37*). The general public concern on chemical additive and food safety has stimulated a continuing search for new natural antioxidants.

The possibility of natural antioxidants replacing/beyond synthetic antioxidants

Increasing attention has turned to the possibility of natural antioxidants replacing synthetic antioxidants in the application of food systems. For examples, sage, mace and black pepper were used to inhibit the oxidation of frozen meat (*38*). Galangal and rosemary extracts have been found to possess significant antioxidant properties. The former was found to improve the oxidative stability and also extended the shelf-life of minced beef (*39*). The later exhibited the ability to inhibit the formation and decomposition of hydroperoxides in bulk corn oil and corn oil-in-water emulsion (*28*). Tomato powders were demonstrated to inhibit the singlet oxygen-catalyzed oxidation of

a-linolenic acid and copper-induced lipid peroxidation (*40*). Meanwhile, plants such as turmeric species, passion fruits, Swiss chards, Canadian prairies, and various vegetables have also been studied for their potential applications in foods as natural sources of antioxidants (*41-44*). Tea is one of the very few materials that contains a very high content of polyphenolics. Extracts of tea have become commercially available in recent years as antioxidants to control deterioration of food lipids (*45*). These natural antioxidants may also protect DNA, protein, and membrane lipids from oxidative damage in biological systems and provide additional health benefits for disease prevention and health promotion (*46*). Phenolics extracted from curcumin and clonal herbs have been investigated in potential medicinal application as antioxidants and anti-inflammatory compounds (*47-49*). Increasing attention has been incorporated to health-promoting and protective effects of vegetable and fruits due to their high content of natural antioxidants (*50-52*). New antioxidants are highly demanded since antioxidants with suitable physicochemical properties are required for individual food or oil systems as well as for various health benefits.

Wheat is an important agricultural commodity

Wheat is a major cereal food crop consumed in many parts of the world and accounts for one-third of the total grain production worldwide (*53*). Today, wheat is grown on more land area than any other commercial crop and continues to be the most important food grain source for humans. Its production leads all crops, including rice, maize, and corn (54). According to the wheat outlook report (*55*), globe wheat production in 2004/05 reached 618 million tons. World trade of wheat is greater than for all other crops combined (*54, 55*). Most wheat varieties cultivated today are categorized as

common wheat and account for approximately 95% of the world's production, while durum wheat comprises nearly all of the rest (*55, 56*). Wheat is divided into two quality classes: hard and soft according to its agronomic and end-use attributes which is based on its suitability for baking bread. Hard wheat contains flour with higher percentage of gluten proteins and is used in the production of common loaf breads and some types of wheat noodles. However, soft wheat is used for a wide diversity of end uses including cookies, cakes, crackers, biscuits, pretzels, soup thickeners and batters (*57; 58*). Direct consumption of wheat products is thought to make up about 20% of the energy supplied in the worldwide total human diet (*59*). Of all the wheat grain consumed, it has been estimated that about 65% is used directly as food for humans (*60*).

Wheat is a potential source of natural antioxidants

Some wheat varieties and cultivars might contain significant levels of dietary antioxidants including phytic acid, phenolic acids, flavonoids, tocopherols and carotenoids (*61- 67*). They primarily exist as substituted benzoic and cinnamic acid compounds, and the total amount may approach 500 mg/kg (*64*). Phenolic acids such as hydroxybenzoic acids and hydroxycinnamic acids in wheat may occur in the free form, but are mostly glycosylated with different sugars, especially glucose. Onyeneho and Hettiarachchy (*68*) reported that ferulic, vanillic, and p-coumaric acids were major phenolics in wheat bran extracts, along with other free phenolics including caffeic, chlorogenic, gentisic, syringic, and p-hydroxybenzoic acids. It is widely accepted that phenolic compounds significantly contribute to the overall antioxidant properties of wheat. Phenolic acids have been strong inhibitors of carcinogenesis at the initiation and promotion stages induced by different compounds (*69*). Carotenoids detected in wheat

included lutein, zeaxanthin, and β-crypoxanthin which varied greatly among different varieties (*63, 65*). The carotenoids particularly lutein and zeaxanthin are believed to be important in preventing ocular damage because they are found in the pigment of the macular region of the normal retina (*70, 71*). It has been hypothesized the dietary supplements with carotenoids may protect the retina and/or retard the progress of AMD. Antioxidant activities were also detected in some wheat and wheat-based food products (*64, 68, 72*). Wheat (winter cultivar Almari and spring cultivar Henika) extracts suppressed radical-induced liposome lipid peroxidation and showed radical cation scavenging activity (*64*). Extracts from Durum wheat (Triticum durum) inhibited oil oxidation using an active oxygen method (*68*). Extracts prepared from three hard winter wheat varieties (Triticum aestivum) were shown to directly react with and quench free radicals, and chelate Fe^{2+} as determined by spectrophotometric and electron spin resonance (ESR) spectrometry methods. Martinez-Tome et al (*73*) reported that some wheat bran commercially available in Spain displayed significant scavenging activity against peroxyl and hydroxyl radicals as well as hydrogen peroxide. Specify hard white wheat (Trego) extracts exerted similar lipid inhibitory effect with α- tocopherol by measuring the oxidative stability index (OSI) (*29*). These finding showed that wheat could be an important source of natural antioxidants for use in functional foods and other applications.

Antioxidant is a value-added factor of wheat and wheat based products

Lipid oxidation is one of the primary causes of quality deterioration in food products, especially in high fat foods including meat products. Wheat or wheat bran extracts have demonstrated the capacity to inhibit lipid oxidation in fish oil (*29*), olive oil,

butter (*73*), and soy oil (*68*). Phytic acid from wheat extract has also been found to inhibit lipid oxidation through its ability to chelate and inactivate prooxidant metals (*62*). These researches suggested the potential of wheat antioxidants as a value-adding ingredient for food quality and safety improvement. Certain case-control and prospective studies showed that there was a reduced risk for colorectal cancer associated with higher intake of whole grains (*74, 75*). Zoran et al. (*76*) reported that wheat bran diet reduced the incidence of colon tumors in rats given azoyxmehtane (AOM). Further, Reddy et al demonstrated the inhibition of tumors was mainly attributed to lipid fraction of wheat bran (*77*). Dietary supplementation with wheat bran also protected against the formation of colonic aberrant crypt foci (ACF) induced by 2-amino-3-mehtylimidaze [4, 5-f] quinoline (IQ) in the rat (*78, 79*). These findings have subsequently led to interest in the association between reduced cancer risk and cereal fiber intake. However, recent human studies did not support the protection by dietary fiber against colorectal cancer or adenoma in nearly 90,000 nurses during 16 years of follow-up as well as another study involving 1303 people. These inconsistent results indicated that other components, especially some antioxidants including lignans, phytic acid, tocopherols, carotenoids and various phenolic components) may contribute to the inhibition against endogenous and exogenous carcinogens and mutagens (*80, 81*). Therefore, wheat antioxidants may add extra value for wheat and wheat based products by human health promotion.

Demonstration of antioxidant properties of wheat may enhance their value-added consumption.

Annual wheat consumption in America has remained relative stable with around 195 pounds per capita (*53*). Wheat is a major staple of the human diet which is primarily

source of whole grain especially in western society. However, the attention paid to grain consumption has been little compared to that for fruits and vegetables, although nutritional guidelines put grains and grain products at the base of the food guide pyramid to emphasize their importance for optimal health. In the United States, 9 out of 10 people are not getting the recommended 3 servings of whole grains each day. Americans consume less than 1 serving per day of whole grain and only 2.5% of total energy intake is from whole grain foods (*82, 83*). On the other hand, there is a current depression in the U.S. wheat market, and the increasing competition from other crops. The U.S. wheat breeding area has been gradually decreasing, and in 2005 the total crop area fell into the lowest record since 1972. In the Northern Plains, increases in other oilseed acreage, especially sunflower seed, and dry peas are expected to curb spring wheat area U.S. wheat production is estimated at 118 million tons for 2004/05, down 10 million tons from 2003/04 as both area and yield dropped (*55*). Wheat producer are looking for value-added opportunities for marketing wheat grain. It is apparent that the utilization of wheat and wheat based products could be enormously improved if consumers could be motivated by established health benefits from consumption. However, research on wheat traditionally focused on improving yield, disease and pest resistance, and functional characteristics in food products, with little attention given to improving nutritional value. Studies are needed to investigate possible value-adding factors and provide more insights about how and why wheat exerts health benefits which may provide fundamental information to exploit this huge potential market. Wheat antioxidants could be potent value-added factor for wheat and wheat base products. Therefore, the studies on wheat antioxidants may

significantly contribute to agricultural economy by promoting value added production and utilization of wheat for health promotion and food manufactures applications.

Methodology for antioxidant characterization

Due to the chemical diversity of antioxidant compounds present in foods, it is unrealistic to separate each antioxidant component and study it individually. In addition, levels of single antioxidants in food do not necessarily reflect their total antioxidant capacity because of the possible synergistic interactions among the antioxidant compounds in a food mixture. Methods of assessing antioxidant capacity fall into two broad categories reflecting the focus on radicals scavenging activity/lipid inhibition in vitro and bioactivity in vivo. The methodology for antioxidant efficiency in vivo generally measures the protection of DNA, lipid and protein from oxidative damage by reactive species (*84, 85*). This is most done by measurement of urinary excretion of DNA base oxidation products, especially 8-hydroxy-2-deoxyguanosine (8OHdG) (*85*). Assessment of total lipid peroxidation is another common approach for antioxidant capacity evaluation in vivo (*86*). In this section, the antioxidant measurements in vitro were briefly discussed by 1. radicals scavenging capacity, 2. Inhibition of lipid oxidation.

Radical scavenging capacity

Currently, the most widely used methods for measuring antioxidant activity are those that involve the generation of radical species. These approaches have been applied to the estimation of antioxidant activity in aqueous systems, but not as much for lipid-soluble antioxidants in nonpolar system (*87-90*). Based on the chemical reactions involved, the major antioxidant capacity assays can be roughly divided into two categories: (1) hydrogen atom transfer (HAT) reaction based assays and (*2*) single electron transfer (ET)

reaction based assays. The majority of HAT-based assays apply a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds. ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced. The degree of color change is correlated with the sample's antioxidant concentrations. HAT- and ET-based assays are intended to measure the radical (or oxidant) scavenging capacity, instead of the preventive antioxidant capacity of a sample (*91*).

The oxygen radical absorbance capacity (ORAC) method developed by Cao et al (*92*) measured the ability of antioxidants to protect protein from damage by free radicals which is a HAT-based assay. In this assay, different generators are used to produce different radicals like peroxyl radical, hydroxyl radical. The method, however, recently adopted the peroxyl radical as standard radical since it is the most common in biological systems (*93*). A major advantage of ORAC is that the method is automated and largely standardized. Also, the ORAC method is reported to mimic antioxidant activity of phenols in biological systems better than other methods since it uses biologically relevant free radicals and integrates both time and degree of activity of antioxidants (*92-94*). 2,2-diphenyl-1picrylhydrazyl (DPPH) radical was one of most commonly used radical for antioxidant capacity evaluation which was a typical ET-based assay. The end point of this assay relies on the decreasing absorbance when DPPH radicals are quenched by antioxidants. The advantages of this assay include DPPH is a stable radical and test is inexpensive and easy to use. However, this assay is sensitive to pH and also reaction was fairly slow, hence it is difficult to obtain absolute antioxidant values. Moreover, DPPH

radical are foreign to biological systems which make it difficult in vivo estimation. The same was true for the 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical which could be generated by chemical and enzymatic pathway. The ABTS assay is rapid and pH stable, however, ABTS is not stable and it is hard to compare values across laboratories (*88*). Overall, 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 F-C reagent) conditions. The pH values have an important effect on the reducing capacity of antioxidants. At acidic conditions, the reducing capacity may be suppressed due to protonation of antioxidant compounds, whereas in basic conditions, proton dissociation of phenolic compounds would enhance a sample's reducing capacity.

Inhibition of lipid peroxidation

Lipid peroxidation is a complex process and occurs in multiple stages. It is well accepted that antioxidants retard lipid peroxidation in foods and biological samples. Hence, many techniques are available for measuring the oxidation rate of membranes, food lipids, lipoproteins, and fatty acids, which are particularly useful for antioxidant evaluation. However, each technique measures something different, and no one method can be said to be the gold standard for lipid peroxidation measurement. Lipid substrates that can be used include emulsions, or liposomes made from fatty acids or fatty acid esters. Oils and melted fats, ground meat, or other food homogenates can also be used. The effect of an antioxidant on lipid peroxidation can be measured mainly by three subcategories: 1. loss of substrates; 2. peroxide assays; 3. determination of end products. Lipid substrates that can be used include emulsions of, or liposomes made from, fatty

acids or fatty acid esters. Oils and melted fats, ground meat, or other food homogenates can also be used. Biological systems can include erythrocytes, isolated lipoproteins (most often low-density lipoproteins or high-density lipoproteins). Methods for loss of substrates include analysis of fatty acids by GC or HPLC. These chromatographic techniques are particular useful for assessing lipid peroxidation stimulated by different pro-oxidants that give different product distribution. The other substrate for peroxidation is oxygen, hence, measurement of the rate of oxygen uptake is another overall index of peroxidation. The uptake of oxygen could be measured by oxygen electrode or more precisely by ESR. This technique is useful when spectrophotometric interference occurs or toxic chemicals interfere with enzymatic technique.

Peroxide assays included total peroxide value measurements and separation of peroxide intermediates. Iodine liberation is one of oldest methods methods used in food industry to evaluate total lipid peroxide (*95*). Fox (ferrous oxidation xylenol orange) assay, glutathione peroxidase (GPX), and cyclooxygenase are also commonly used as peroxide assays (*96, 97*). The amount of peroxide present at a given time during lipid peroxidation depends on both the rate of initiation of peroxidation and on how quickly peroxides break down. Lipid peroxides are fairly stable at room temperature, but break down quickly following heating to give a complex mixture of aldehydes and other products (*96*). Measurement of end products of lipid peroxidation is another strategy to evaluate antioxidant function. The thiobarbituric acid (TBA) test is one of the oldest and most frequently used tests for lipid peroxidation, and measures the end product of TBA-reactive substance (TBARS) (*98*). The advantage of the test is that it is simple to run. however, many other compounds interfere with the results aside from malondialdehyde

(end product during peroxidation of lipid-containing systems) and results varied significantly according to different test conditions. To avoid these problems, several HPLC methods have been applied to separate the $(TBA)_2$-MDA adduct from other chromogens (*99*). In summary, there is no standard method to measure lipid peroxidation, either in foods or in biological material. The most specific assays of lipid peroxidation involve HPLC, and GC-MS based determinations of individual products as specific aldehydes (MDA) or peroxides.

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Chapter 2. Comparison of Grain, Bran, Aleurone and Micronized Aleurone for Their Antioxidant Properties

ABSTRACT

Swiss red wheat grain, bran, aleurone, and micronized aleurone were examined and compared for their free radical scavenging properties against 2,2-diphenyl-1-picryhydrazyl radical (DPPH·), radical cation $ABTS^{\bullet+}$, peroxide radical anion $O_2^{\bullet-}$ and oxygen radical (ORAC), chelating capacity, total phenolic content (TPC), and phenolic acid composition. The results showed that micronized aleurone, aleurone, bran, and grain may significantly differ in their antioxidant properties, TPC, and phenolic acid composition. Micronized aleurone had greatest antioxidant activities, TPC, and concentrations of all identified phenolic acids, suggesting the potential post-harvest treatment on antioxidant activities and availability of TPC and phenolic acids. Ferulic acid was the predominant phenolic acid in Swiss red wheat and accounted for about 57-77 % of total phenolic acids on a weight basis. Ferulic acid concentration was well correlated with scavenging activities against radical cation and superoxide anion, TPC, and other phenolic acid concentrations, suggesting the potential use of ferulic acid as a marker of wheat antioxidants. In addition, the ORAC value of 50% acetone extracts was 3-20 folds greater than that of the ethanol extracts, indicating that 50% acetone may be a better solvent system for monitoring antioxidant properties of wheat. These data suggest the possibility to improve the antioxidant release from wheat-based food ingredients through post-harvest treatment or processing.

INTRODUCTION

Severe oxidative stress, a result of imbalance between antioxidant defense system and the formation of reactive oxygen species, may damage life important membrane lipids, proteins, DNA, and carbohydrates (*1, 2*). The damage may cause cell injury and death, and exacerbate the development of several aging-related chronic diseases including cancer and heart disease (*1*). It has been well accepted that dietary antioxidants may prevent these physiologically important molecules from oxidative damages, and consequently reduce the risk of aging-related diseases and/or promote general human health (*2 - 4*). Significant antioxidative activities have been detected in several wheat samples, including spring cultivar Henika (macaroni) wheat (*Triticum durum)* (*5*), winter cultivar Almari and spring cultivar Henika (*Triticum aestivum*) (*6*), hard winter wheat cultivars Akron, Trego and Platte (*Triticum aestivum) (3, 7, 8*), and blue aleurone spring wheat (*Triticum aestivum)* (*9*). Wheat antioxidants are capable to directly react with and quench free radicals, reduce the availability of transition medals (chelating activity), and suppress lipid peroxidation in fish oil and liposome (*3, 5 - 8*). It is widely accepted that phenolic compounds significantly contribute to the overall antioxidant properties of wheat. Onyeneho and Hettiarachchy (*5*) reported that ferulic, vanillic and p-coumaric acids were major phenolics in wheat bran extracts, along with other free phenolics including caffeic, chlorogenic, gentisic, syringic, and p-hydroxybenzoic acids. They noted that phenolic acids are concentrated in bran and aleurone fractions of wheat (*10*). Quantification methods of phenolic acids in wheat flour and grain have been investigated, because phenolic acid content in flour was considered as a potential parameter to monitor the carryover of bran in white flour. The previous studies also showed that antioxidative

properties of wheat might vary among wheat cultivars, and may be significantly altered by growing conditions (*3, 7, 8*).

No research, however, has been performed to compare the antioxidant properties of wheat aleurone and bran. Also how the post-harvest treatment or processing may influence the antioxidant properties of wheat based products. Therefore, the present study was conducted to examine and compare the antioxidant properties of aleurone extracts from Swiss red wheat with that of grain, bran, and micronized aleurone. This research is part of our continuous efforts to promote the improved production and utilization of value-added wheat for health promotion and disease prevention.

MATERIALS AND METHODS

Materials. Swiss red wheat grain, bran, aleurone, and micronized aleurone were provided by Buhler AG (Uzwil, Switzerland). 2,2'-bipyridyl, disodium ethylenediaminetetracetate (EDTA), 2,2-diphenyl-1-picryhydrazyl radical (DPPH·), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, fluorescein (FL), lauryl sulfate sodium salt, hypoxanthine (HPX), xanthine oxidase (XOD), nitro blue tetrazolium solution (NBT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), α-tocopherol (Vit E), ascorbic acid (Vit C), and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (St. Louis, MO), while 2,2'-azobis (2-amino-propane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA). β-cyclodextrin (RMCD) was purchased from Cyclolab R & D Ltd. (Budapest, Hungary). All other chemicals and solvents were of the highest commercial grade and used without further purification.

Extraction and testing sample preparation. 4 grams of each wheat sample was ground to fine powder using a micro-mill manufactured by Bel Art Products (Pequannock, NJ) and extracted for 15 hours with 40 mL of 50% acetone under nitrogen at ambient temperature or extracted for 2 hours with absolute ethanol using a Soxhlet extractor. The ethanol was evaporated at 30°C under reduced pressure, and the residue was re-dissolved in 7% RMCD solution. The 7% RMCD solutions from the ethanol extracts and the 50% acetone extracts were kept in the dark under nitrogen at room temperature until further analysis.

Radical cation ABTS$^{\bullet+}$ scavenging activity. Radical scavenging capacity of wheat antioxidant was evaluated against ABTS$^{\bullet+}$ generated by the chemical method according to a previously reported protocol (*11*). 50 μL of wheat antioxidants in 50% acetone was diluted with 450 μL of 7% RMCD to obtain the testing samples. ABTS$^{\bullet+}$ was prepared by oxidizing 5 mM aqueous solution of ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt, with manganese dioxide at ambient temperature for 30 min. The ABTS$^{\bullet+}$-antioxidant reaction mixture contained 1.0 mL of ABTS$^{\bullet+}$ with an absorbance of 0.7 at 734 nm, and 80 μL of antioxidant testing sample or 80 μL of 7% RMCD solution for the control. The absorbance at 734 nm was measured at 1 min of the reaction, and the trolox equivalent was calculated using a standard curve prepared with trolox.

Radical DPPH scavenging activity. Free radical scavenging capacity of wheat extracts were determined according to the previously reported procedure using the stable 2,2-diphenyl-1-picryhydrazyl radical (DPPH$^{\bullet}$) (*3*). The final concentration was 100 μM

for $DPPH^{\bullet}$ and the final reaction volume was 2.0 mL. The absorbance at 517 nm was measured against a blank of pure ethanol at 0, 1, 5, 10, 20, 40, 80, and 1400 min and used to estimate the remaining radical levels according to a standard curve. The absorbance measured at 40 min of the antioxidant-DPPH radical reactions was used to compare the DPPH radical scavenging capacity of wheat extracts to that of 50 mM ascorbic acid, BHT, and α-tocopherol. To determine the ED_{50} value of the wheat antioxidants against DPPH radicals, seven levels of each wheat extract were employed. $A_{517\,nm}$ at 80 min of reaction was used to establish the ED_{50} value. ED_{50} value is the concentration of an antioxidant necessary to quench 50% radicals in the reaction mixture under the experimental conditions.

Superoxide anion radical $O_2^{\bullet-}$ scavenging activity. $O_2^{\bullet-}$ scavenging activity was determined using hyoxanthine/xanthine oxidase system following a procedure described previously (*10*). Nitro blue tetrazolium solution (NBT, 0.34mM), hyoxanthine solution (HPX, 2mM), xanthine oxidase solution (XOD, 0.56 unit/mL) were prepared in a sodium phosphate buffer (0.05M; pH 7.4). The reaction mixture contained 0.2 mL of 0.34 mM NBT solution, 0.7 mL of 2 mM HPX solution, 0.1mL of wheat bran antioxidant in 50% acetone, 0.2 mL of 0.56 units/mL XOD solution. The concentration of wheat bran antioxidant in test solution was 8.3 mg bran equivalent/mL.

The decrease in absorbance was measured at 560 nm every minute for a 7 min period, and the % $O_2^{\bullet-}$ remaining was calculated to evaluate the $O_2^{\bullet-}$ scavenging capacity of each bran extract according to the following equation:

$$\%\ O_2^{\bullet-}\ \text{remaining} = \text{Slope of } Abs_{(sample)}/\text{Slope of } Abs_{(control)} * 100$$

The Slope of $Abs_{(sample)}$ was obtained by plotting the $A_{560\ nm}$ of the bran antioxidant-radical reaction against the corresponding reaction time, while the Slope of $Abs_{(control)}$ was determined by plotting the $A_{560\ nm}$ of the control radical reaction containing no antioxidant.

ORAC assay. ORAC assay was conducted using fluorescein (FL) as the fluorescent probe according to a protocol described by Huang and others (*13*). The final assay mixture contained 0.067 μM of FL, 60 mM of AAPH, 300 μL of wheat antioxidants or 7% RMCD for a reagent blank. The fluorescence of an assay mixture was determined and recorded every min for duration of 60min. The trolox equivalent was calculated using a standard curve prepared with trolox, and used to compare ORAC of each antioxidant. All tests were conducted in triplicate.

Chelating activity. Fe^{2+} chelating activity was measured by 2,2'-bipyridyl competition assay (*8*). The reaction mixture contained 0.1 mL of 1 mM $FeSO_4$ solution, 50 μL of wheat antioxidant in 50% acetone, 0.3 mL of 10% hydroxylamine-HCl, 0.4mL of 2,2'-bipyridyl solution, 0.8 mL of Tris-HCl buffer (pH 7.4). The absorbance at 522 nm was measured and used to evaluate Fe^{2+} chelating activity using EDTA as a standard, all tests were conducted in triplicate.

Total phenolic contents. The total phenolic contents of wheat extracts were determined using Folin-Ciocalteu reagent (*3*). The Folin-Ciocalteu reagent was prepared by refluxing a mixture of sodium tungstate, sodium molybdate, 85% phosphoric acid, and concentrated hydrochloric acid for 10 hours, and followed by reacting with lithium sulfate, and oxidizing by a few drops of bromine. The resulting

solution was filtered. In brief, the reaction mixture contained 50 μL of wheat extracts and 250 μL of the Folin-Ciocalteu reagent freshly prepared in our laboratory and 0.75 mL of 20% sodium carbonate and 3 mL of pure water. After two hours of reaction at ambient temperature, the absorbance at 765 nm was measured and used to calculate the phenolic contents using gallic acid as a standard. All tests were conducted in triplicate.

Phenolic acid composition. After removing acetone, the wheat antioxidants were hydrolyzed with 4N NaOH for 4 hours at 55°C under nitrogen, acidified using 6N HCl, and extracted with ethyl ether-ethyl acetate (1:1, v/v) according to the procedure described previously (*14*). The ethyl ether-ethyl acetate was evaporated at 25° using a nitrogen evaporator, and the solid residue was re-dissolved in methanol, filtered through a 0.45 μm membrane filter, and kept in dark under nitrogen until HPLC analysis. Phenolic acid composition in the methanol solution was analyzed using a Shimadzu LC-10A model HPLC, equipped with a photodiode array UV detector, an auto-sampler, and a Phenomenex C18 column (250 mm × 4.6 mm). Phenolic acids were separated using a linear gradient elution program with a mobile phase containing solvent A (acetic acid/H_2O, 2:98, v/v) and solvent B (acetic acid/acetonitrile/ H_2O, 2:30:68, v/v/v). The solvent gradient was programmed from 10 to 100% B in 42 min with a flow rate of 1.5 mL/min (*15*). Identification of phenolic acids was accomplished by HPLC-MS and comparing the retention of peaks in wheat samples to that of the standard compounds.

Statistical analysis. Data were reported as mean ± SD for triplicate determinations. Analysis of variance and least significant difference tests (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL) were conducted to identify

differences among means, while a Pearson Correlation test was conducted to determine the correlations among means. Statistical significance was declared at $P < 0.05$.

RESULTS

Radical cation scavenging activity. 50% acetone extracts of Swiss red wheat grain, bran, aleurone and micronized aleurone were examined and compared for their free radical scavenging activities against radical cation $ABTS^{\bullet+}$. All extracts showed $ABTS^{\cdot+}$scavenging capacity (Table 2.1). The greatest $ABTS^{\bullet+}$ scavenging capacity was detected in micronized aleurone extract, followed by aleurone, bran and grain, respectively (Table 2.1). Aleurone (Asp1 and Asp2), isolated from the bran, had greater $ABTS^{\bullet+}$ scavenging capacity than bran, suggesting that antioxidants are concentrated in aleurone. Significant differences in their radical cation scavenging activities were detected among wheat samples, except between aleurone sample 2 (Asp2) and the micronized aleurone prepared from the aleurone sample 1 (Asp1). Micronizied aleurone (mAsp1) exhibited a significantly stronger $ABTS^{\bullet+}$ scavenging capacity than that of the bran (Asp1), suggesting that post-harvest treatment may alter the antioxidant activities of wheat. For all the tested 50% acetone extracts, $ABTS^{\bullet+}$ scavenging capacity was correlated to $O_2^{\bullet-}$ scavenging activity ($r = 0.96$, $P = 0.009$), ORAC ($r = 0.97$, $P = 0.05$), chelating capacity against Fe^{2+} ($r = 0.92$, $P = 0.03$) and the total phenolic contents ($r = 0.95$, $P = 0.014$). In addition, the $ABTS^{\bullet+}$ scavenging capacity was also correlated with the DPPH radical scavenging capacity of the ethanol extract ($r = 0.94$, $P = 0.02$).

Table 2.1. Free Radical Scavenging Properties of Swiss Red Wheat Fractions*

	% $O_2^{\bullet-}$ Remaining	$ABTS^{\bullet+}$ (TE μmoles/g wheat)	ED_{50} for DDPH (mg/mL)
Grain	77.56a ± 3.17	14.67a ± 0.48	20
Bran	59.19b ± 0.13	19.74b ± 0.17	9.1
Asp1	58.62b ± 1.77	22.56c ± 0.22	8.8
Asp2	44.31c ± 0.08	24.29d ± 0.50	8
mAsp1	43.13c ± 0.37	24.47d ± 0.24	6.2

* Grain, bran, Asp1, Asp2, and mAsp1 stand for Swiss red wheat grain, bran, aleurone sample 1, aleurone sample 2, micronized aleurone prepared from aleurone sample 1, respectively. TE stands for trolox equivalent. Within each column, means with the same letter are not significantly different ($P < 0.05$). ED_{50} is the concentration of wheat extracts to quench 50% of DPPH radicals in the reaction mixture.

Superoxide anion radical $O_2^{\bullet-}$ scavenging activity. $O_2^{\bullet-}$ scavenging activity of wheat samples was evaluated using the 50% acetone extracts and expressed as % $O_2^{\bullet-}$ remaining. Micronized aleurone showed the greatest $O_2^{\bullet-}$ scavenging activity, while grain had the lowest activity (Table 2.1). The relative $O_2^{\bullet-}$ scavenging activity of the 50% acetone extracts was micronized aleurone > aleurone > bran > grain, on a per weight basis. The $O_2^{\bullet-}$ scavenging activity of aleurone was significantly greater than that of the bran used to isolate the aleurone, suggesting the potential effect of post-harvest processing on antioxidant property of wheat. Correlation was detected between $O_2^{\bullet-}$ scavenging activity and ORAC (r = 0.94 and $P = 0.027$), $ABTS^{\bullet+}$ scavenging capacity, chelating activity (r = 0.96, $P = 0.01$), $DPPH^{\bullet}$, and TPC (r = 0.97, $P = 0.01$).

Radical DPPH scavenging activity. The ethanol extracts were analyzed and compared with α-tocopherol, BHT, and ascorbic acid for free radical scavenging activity against stable $DPPH^{\bullet}$. All wheat extracts, at a concentration of 12.5 mg wheat equivalent/mL, were capable to directly react with and quench $DPPH^{\bullet}$ (Figure 2.1, Table 2.1), although their $DPPH^{\bullet}$ scavenging activities were weaker than that of 50 mM α-tocopherol, BHT, or ascorbic acid. At 40 min of reaction, micronized aleurone quenched the greatest amount of $DPPH^{\bullet}$ in the system, and followed by aleurone, bran, and grain, respectively (Figure 2.1), on a per weight basis. The concentration required to scavenge 50% of the free radicals in the reaction mixture, the ED_{50} value, was also determined. The order of the ED_{50} values was micronized aleurone < aleurone < bran < grain (Table 2.1). Smaller ED_{50} value corresponds to a greater $DPPH^{\bullet}$ scavenging activity. These data indicated that radical scavengers are concentrated in the aleurone fraction of wheat bran, and reduction of particle size might increase the availability of wheat antioxidants. Similar dose and time effects were observed in all wheat antioxidant-DPPH radical reactions, and the dose and time effects of micronized aleurone extract were reported in Figure 2.2 as an example. DPPH radical scavenging activity of the ethanol extracts was not correlated with the ORAC of ethanol extracts. Interestingly, DPPH radical scavenging activity of the ethanol extracts was correlated with the $ABTS^{\bullet+}$ scavenging capacity ($r = 0.94$, $P = 0.02$), $O_2^{\bullet-}$ scavenging activity ($r = 0.92$, $P = 0.03$), ORAC ($r = 0.97$, $P = 0.05$), chelating capacity against Fe^{2+} ($r = 0.94$, $P = 0.01$) and the total phenolic contents ($r = 0.90$, $P = 0.04$) of the 50% acetone extracts.

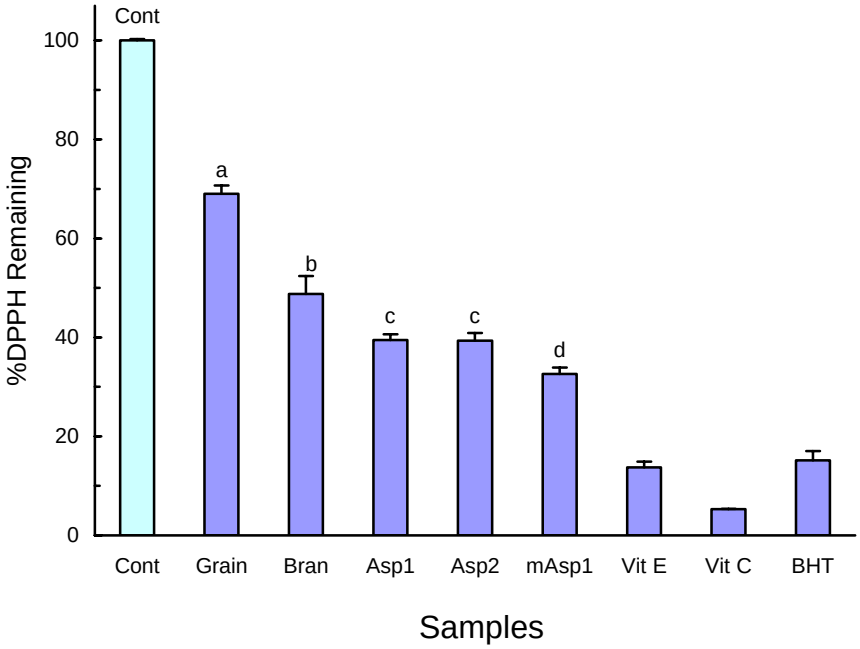

Figure 2.1. Radical DPPH scavenging activity.

Grain, Bran, Asp1, Asp2, mAsp1 represent Swiss red wheat grain, bran, aleurone sample 1, aleurone sample 2, and micronized aleurone prepared from the aleurone sample 1, respectively, while the Cont represents the control containing no antioxidant. The final DPPH radical concentration was 100 μM in all reaction mixtures, while the final concentration of wheat extracts was 12.5 mg wheat equivalent per mL. Vit E, Vit C and BHT represent α-tocopherol, ascorbic acid, and butylated hydroxytoluene, respectively, at a final concentration of 50 mM. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P <$ 0.05).

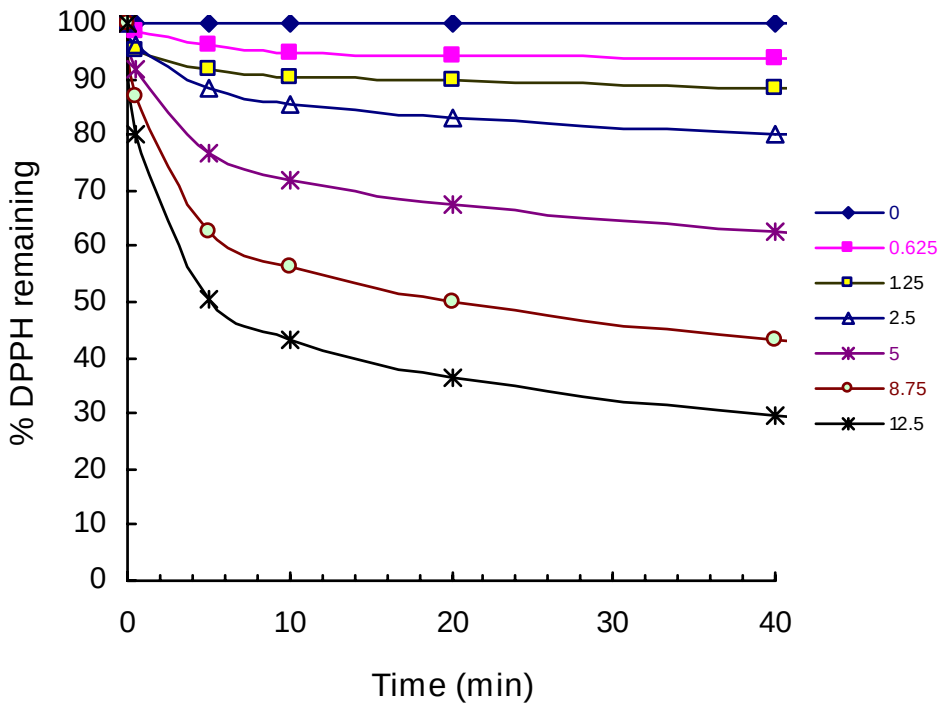

Figure 2.2. Reaction kinetics of wheat extracts with DPPH radical.

0, 0.625, 1.25, 5, 8.75, and 12.5 represent the final antioxidant concentration of 0, 0.625, 1.25, 5, 8.75, and 12.5 mg wheat equivalent/mL in the reaction mixtures. All tests were conducted using the ethanol extracts. The final DPPH radical concentration was 100 μM in all reaction mixtures.

ORAC assay. ORAC values were determined for both 50% acetone and ethanol extracts, and expressed as trolox equivalent (TE). Both ethanol extracts and 50% acetone extracts exhibited significant ORAC (Table 2.2). The greatest ORAC value was observed in Asp1 among ethanol extracts, and followed by that of Asp2, micronized Asp1 and bran. This order differed to that detected among the 50 % acetone extracts. Furthermore, no correlation in the ORAC values was detected between ethanol extracts and 50% acetone extracts. These data suggested the potential effects of extraction

solvent on antioxidant activity estimation. The TE value of 50% acetone extract was 3-20 fold greater than that of the corresponding ethanol extract (Table 2.2), suggesting that a larger portion of wheat antioxidants are bound and has higher polarity. Wheat fractions differed in their ORAC values regardless of extraction solvent, indicating that antioxidants are mainly distributed in the aleurone fraction of wheat bran. The ORAC value of 50% acetone extracts was correlated with $O_2^{\bullet-}$ scavenging activity, $ABTS^{\bullet+}$ scavenging capacity, $DPPH^{\bullet}$ scavenging capacity, and chelating activity ($r = 0.94$, $P = 0.02$). No correlation between ORAC value and TPC was observed.

Table 2.2. ORAC of Swiss Red Wheat*

	ORAC (TE μmoles/g wheat)	
	Ethanol extract	50% Acetone extract
Grain	N/A	51.46a ± 6.54
Bran	5.068a,u ± 1.73	107.53b,v ± 4.1
Asp1	40.48b,x ± 2.27	125.18c,y ± 13.4
Asp2	37.08b or c, w ± 1.95	136.42c, z ± 9.53
mAsp1	34.47c, s ± 2.33	126.48c, t ± 6.89

* Grain, bran, Asp1, Asp2, and mAsp1 stand for Swiss red wheat grain, bran, aleurone sample 1, aleurone sample 2, and micronized aleurone prepared from aleurone sample 1, respectively. ORAC stands for the oxygen radical absorbance capacity. Same letter of a, b, and c indicates the means within came column are not significantly different ($P < 0.05$), while same letter of u, v, w, x, y, and z indicates the means within same row are not different at $P < 0.05$ (n = 3).

Chelating activity of Swiss wheat extracts. The chelating properties of the 50% acetone extracts were examined against Fe^{+2} and reported as EDTA equivalents (Figure 2.3). The grain had significantly lower chelating activity than bran and aleurone fractions, but bran and aleurone isolated from the bran showed similar chelating activity (Figure 2.3). Reduction in particle size did not result in further increase in the chelating activity of aleurone. The chelating activity was correlated with $O_2^{\bullet-}$ scavenging activity, $ABTS^{\bullet+}$ scavenging capacity, $DPPH^{\bullet}$ scavenging capacity, and ORAC value, but not with total phenolic contents under the experimental condition.

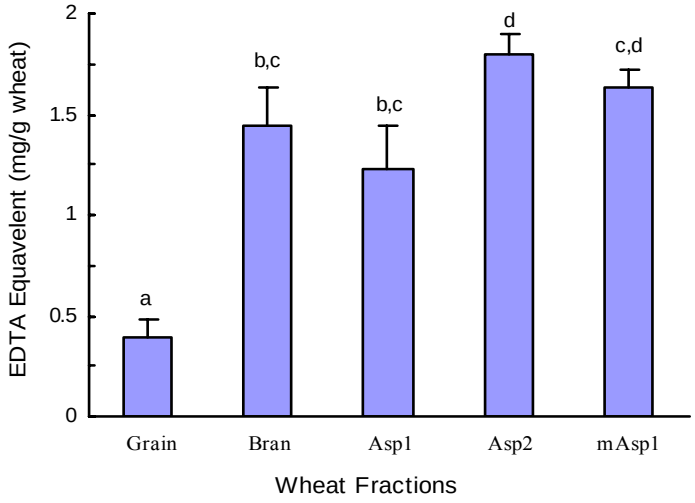

Figure 2.3. Chelating capacity of wheat extracts.

The chelating activities of wheat extracts were expressed as EDTA equivalent. Grain, Bran, Asp1, Asp2, mAsp1 represent Swiss red wheat grain, bran, aleurone sample 1, aleurone sample 2, and micronized aleurone prepared from the aleurone sample 1, respectively. All tests were conducted using the 50% acetone extracts. The vertical bars represent the standard deviation (n=3), and values marked by the same latter are not significantly different ($P < 0.05$).

Total phenolic content. The Swiss red wheat samples were examined for their total phenolic contents (TPC) expressed as gallic acid equivalent (GE). Five wheat samples differed from each other in their TPC (Figure 2.4). The greatest TPC of 4.04 mg GE/g wheat was detected in the micronized aleurone, while the grain had the lowest TPC value of 1.8 mg GE/g wheat. TPC was correlated with $O_2^{\bullet -}$, $DPPH^{\bullet}$ scavenging capacity and $ABTS^{\bullet +}$ scavenging capacity, but not ORAC ($r = 0.87$ and $P = 0.055$) or chelating activity ($r = 0.87$ and $P = 0.053$).

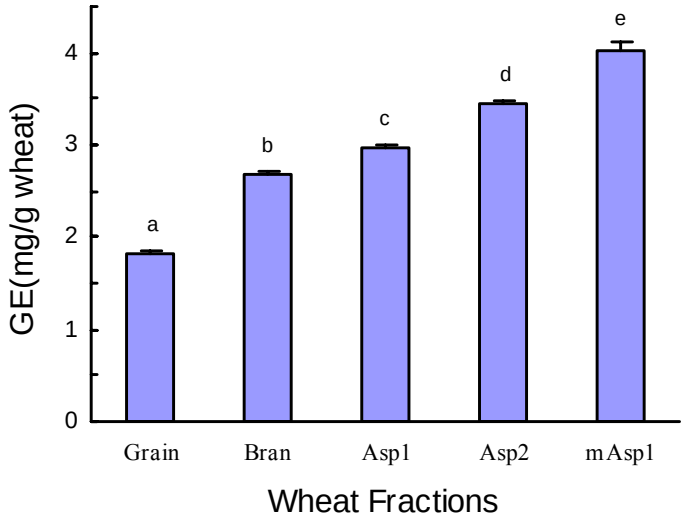

Figure 2.4. Total phenolic contents of wheat extracts.

Grain, Bran, Asp1, Asp2, mAsp1 represent Swiss red wheat grain, bran, aleurone sample 1, aleurone sample 2, and micronized aleurone prepared from the aleurone sample 1, respectively. All tests were conducted using the 50% acetone extracts. The vertical bars represent the standard deviation (n=3). Values marked by the same letter are not significantly different ($P < 0.05$).

Phenolic acid composition. Five phenolic acids, including ferulic, syringic, p-benzoic, vanillic, and coumaric acids, were detected in the wheat extracts (Table 2.3). Ferulic acid was the predominant acid in all extracts and accounted for about 57-78 % of the total identified phenolic acids on a per weight basis. Aleurone samples had the greatest concentration of each detected phenolic acids, and followed by that of bran and grain, suggesting that phenolic acids are concentrated in the aleurone fraction of Swiss red wheat bran (Table 2.3). The two aleurone samples isolated from the same bran contained different amount of phenolic acids, indicating the potential influence of aleurone isolation procedure on the antioxidant properties of aleurone. Micronized aleurone (mAsp1) had significantly greater concentration of each detected phenolic acid than the aleurone (Asp1) (Table 2.3), although a similar ratio of ferulic, syringic, p-hydroxy benzoic, vanillic and coumaric acids was observed in both extracts. These results suggested that micronization increased the availability of phenolic acids under the experimental conditions.

In addition, ferulic acid concentration in wheat samples was correlated with scavenging capacities against $ABTS^{\bullet+}$ and $O_2^{\bullet-}$, as well as total phenolic contents. The correlation coefficient (r) was 0.88 ($P = 0.05$), 0.91 ($P = 0.03$), and 0.98 ($P = 0.003$) between ferulic acid concentration on a per weight basis and $ABTS^{\bullet+}$ scavenging activity, $O_2^{\bullet-}$ quenching capacity , or total phenolic content of the 50 % acetone extracts, respectively. Ferulic acid concentration was also highly correlated with all other identified phenolic acids in the wheat extracts. The correlation coefficient was 0.92-0.98 ($P < 0.01$) between ferulic acid concentration and the concentration of p-OH benzoic,

vanillic, or coumaric acids, and was 0.97 between ferulic acid and syringic acid levels (P = 0.02).

Table 2.3. Phenolic Acid Composition of Swiss Red Wheat*

	p-OH Benzoic (μg/g wheat)	Vanillic (μg/g wheat)	Syringic (μg/g wheat)	Coumaric (μg/g wheat)	Ferulic (μg/g wheat)
Grain	5.0a ± 0.2	4.9a ± 0.2	13.7a ± 0.3	1.9a ± 0.1	33.7a ± 1.3
Bran	19.7b ± 0.3	16.5b ± 0.3	57.2b ± 0.5	9.0b ± 0.0	209.3b ± 0.3
Asp1	24.2c ± 4.1	19.7c ± 0.7	69.3c ± 0.6	8.2c ± 0.0	279.7c ± 0.4
Asp2	28.4c ± 0.1	20.0c ± 0.4	90.3d ± 0.1	10.6d ± 0.0	373.6d ± 0.7
mAsp1	38.8d ± 3.5	29.6d ± 0.6	97.7e ± 0.8	14.7e ± 0.1	625.7e ± 3.7

* Grain, bran, Asp1, Asp2, and mAsp1 stand for Swiss red wheat grain, bran, aleurone sample 1, aleurone sample 2, and micronized aleurone prepared from aleurone sample 1, respectively. The analysis was conducted using the 50% acetone extracts. p-OH benzoic, Vanillic, Syringic, Coumaric, and Ferulic stand for p-OH benzoic, vanillic, syringic, coumaric, and ferulic acids, respectively. Within each column, means with the same letter are not significantly different ($P < 0.05$, n = 3).

DISCUSSION

It has been widely accepted that diet may significantly influence human health and life quality. Recently, more consumers are interested in food products that either reduce the risk of, or manage, a specific health condition (*16*). To achieve the maximum benefits from diet, it is critical to understand the bioactive factors and their distribution in food ingredients, as well as the effects of food formula, food processing and storage on

availability of these beneficial components. This understanding is also important for improving the safety and quality of consuming functional foods, because of the interactions among food components and foods, as well as the potential interactions between foods, and supplements or medicines. In addition, this understanding may lead to an improved utilization and application of agricultural products including wheat, and enhance the agricultural economy. For instance, wheat grain may be further processed into flour, bran, aleurone and micronized aleurone, and used to prepare food products, which may have different requirements in sensory properties, quality and stability, and health benefits, for different groups of consumers. This research is part of our continuous effort to promote the value-added production and utilization of wheat in improving human nutrition for disease prevention and health promotion.

In this study, grain, bran, and aleurone isolated from bran were compared for their antioxidant properties, total phenolic contents, and phenolic acid composition. Aleurone had the greatest radical scavenging activities, total phenolic contents, and phenolic acid concentration on a per weight basis under the experimental conditions, and followed by bran and grain, respectively. These results, in agreement with previous reports (*5, 17, 18*), demonstrate that antioxidants including phenolics are concentrated in the aleurone fraction of wheat bran, suggesting the potential to further isolate the aleurone fraction from bran and use as concentrated dietary source of natural wheat antioxidants. The remaining bran, low in phenolics, may be used as a food ingredient for dietary insoluble fiber. This bran ingredient may be used in making some food products (including Chinese noodle) rich in fiber with less concern about color instability, which is caused by the enzymatic browning reaction of phenolics. In other words, understanding of the

distribution of bioactive components in wheat may lead to a value-added production and consumption of wheat and wheat-based products. This will benefit both consumers and wheat producers.

It was noted in this research that micronization, a post-harvest treatment, increased antioxidant activities, total phenolic content, and phenolic acid concentration of aleurone. This may be explained by the increased surface area through micronization. Micronization reduced the particle size of aleurone and consequently increased the total particle surface area on a per weight basis. Enlarged surface area increased the amount of extractable antioxidants. This observation suggests potential effects of post-harvest treatment and processing on the overall antioxidant properties of wheat products. This observation was supported by an earlier research observation that pearling time may alter the antioxidant and phenolic content in the pearling fractions (*19*). It is also noted that only one wheat variety was employed in this research. More research is required to further investigate post-harvest treatment and processing on the antioxidant capacity of wheat products using other varieties.

In addition, it was noted that the ORAC value of 50% acetone extracts was 3-20 times greater and better correlated with other antioxidant activities, total phenolic contents, and phenolic acid concentrations, than that of the ethanol extracts. This may be partially explained by the procedure used to prepare the ethanol extracts for the ORAC test. The ethanol was removed from the ethanol extracts, and the residue was re-dissolved in 7% RMCD. The resulted 7% RMCD solution was centrifuged and the clear supernatant was used for ORAC assay. Low polarity compounds and the compounds hard to be re-dissolved were lost in the solid precipitates. This may result in the

reduction in ORAC values. Furthermore, these data also indicate the potential effects of solvent used in antioxidant extraction on results of antioxidant activity tests. Several solvent systems have been used to extract wheat antioxidants for evaluations of different antioxidative activities. These included ethanol (*3, 7, 8*), pure water (*6, 20*), acetone-water (4:1, v/v), ethanol-water (4:1, v/v), methanol-water (4:1, v/v) (*6*), 95% ethanol (*5*), and 50% acetone in this study. Recently, methanol and 1.0 N HCl (85:15, v/v) were also used to extract total anthocyanin from blue-grained wheat (*9*). It is important for comparing the results obtained from different laboratories to examine and compare the extraction efficacy of each commonly used solvent systems. An ongoing study is being conducted in our laboratory to evaluate a group of solvent systems for their efficacies in wheat antioxidant extraction.

In agreement with the previous observation (*5, 10*), ferulic acid was the predominant phenolic acid detected in Swiss red wheat antioxidant extracts and accounted for 57-78 % of the total phenolic acids on a per weight basis. The grain of Swiss Red wheat contained 33.71 μg of extractable ferulic acid per gram of seeds, which is greater than 5 ppm (μg/g) of free and soluble bound ferulic acid (*21*), but is much lower than the reported typical level of 500 μg of ferulic acid per gram of ground whole wheat (*10, 21*). The extractable phenolic acid is a portion of, and may account for less than 10 % of total phenolic acid presented in wheat (*21*). Ferulic acid has been evaluated for its potential application as an analytical parameter in rapid determination of bran carryover in flour during milling (*22*). Antioxidant properties of ferulic acid have been evaluated and reviewed by Graf (*10*). In this study, ferulic acid content was well correlated with antioxidant activities, total phenolic content, and concentrations of other

identified individual phenolic acids. Therefore, ferulic acid may serve as a marker for quality control of wheat antioxidants or may be used to monitor wheat antioxidant processing.

In conclusion, this study indicates that antioxidants including phenolic acids are concentrated in the aleurone fraction of wheat bran. Further, micronization may increase the antioxidant activities, total extractable phenolic content, and extractable phenolic acid of wheat aleurone. 50% acetone may be a better solvent system than ethanol for monitoring antioxidants in wheat and wheat-based food ingredients. Ferulic acid is the predominant phenolic acid in Swiss red wheat, and accounts for 57-78 % of the total phenolic acids on a dry weight basis. Ferulic acid concentration of all Swiss red wheat samples tested in this study was well correlated with $ABTS^{\bullet+}$ and $O_2^{\bullet-}$ quenching abilities, total phenolic content, and other phenolic acid concentrations, suggesting that ferulic acid may be a potential marker for quality control of wheat antioxidants.

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Chapter 3. Phytochemicals and Antioxidant Properties of Bran samples of Seven Wheat Varieties

ABSTRACT

Bran samples of seven wheat varieties from four different countries were examined and compared for their phytochemical compositions and antioxidant activities. Phenolic acid composition, tocopherol content, carotenoid profile, and total phenolic content (TPC) were examined for the phytochemical composition of wheat bran, whereas the measured antioxidant activities were free radical scavenging properties against 2,2-diphenyl-1-picryhydrazyl radical ($DPPH^{\bullet}$), radical cation $ABTS^{\bullet+}$, peroxide radical anion $O_2^{\bullet-}$ and oxygen radical (ORAC), and chelating capacity. The results showed that the tested wheat bran samples differed in their phytochemical compositions and antioxidant properties. Ferulic acid, with a concentration range of 99 – 231 μg/g, was the predominant phenolic acid in all of the tested bran samples and accounted about 46 –67 % of total phenolic acids on a weight basis. Total tocopherol was 0.92 – 6.90 μmoles per 100 grams of bran, and the concentrations for α-, δ-, and γ- tocopherols were 1.28 – 21.29, 0.23 – 7.0, and 0.92 – 6.90 μg/g, respectively. In addition, lutein and cryptoxanthin were detected in all of the tested bran samples with levels of 0.50 – 1.80 and 0.18 – 0.64 μg/g, respectively. Zeaxanthin was detected in the 6 bran samples, and the greatest zeaxanthin concentration of 2.19 μg/g was observed in the Australia general-purpose wheat bran. β-carotene was detected in 4 of the tested bran samples at a range of

0.09 – 0.40 μg/g. These data suggest that wheat and wheat bran from different countries may differ in their potentials for health promotion and disease prevention.

INTRODUCTION

It has been postulated that antioxidants may modulate cellular oxidative status and prevent biologically important molecules such as DNA, protein, and membrane lipid from oxidative damage, and consequently reduce the risk of several chronic diseases including cancer and cardiovascular disease (*1-3*). Increasing evidence indicates that wheat and wheat-based food products contain significant levels of natural antioxidants, which may provide health benefits to consumers in addition to general nutrients and energy (*3-12*). Wheat is an important agricultural commodity and dietary component across the world. Previous studies showed that wheat varieties vary in their antioxidant properties, total phenolic content, phenolic acid composition, and carotenoid profile (*3-5, 13*). It was also noted that growing conditions and the interaction between environmental factors and genotype altered the antioxidant properties and phytochemical composition of wheat grain and bran (*6, 13*). Recent study of Swiss red wheat grain and fractions (*10*) showed that phenolic antioxidants are concentrated in the aleurone fraction of wheat bran, and further micronization increased the availability of antioxidants in the aleurone samples. Several phenolic acids have been detected in wheat and wheat fractions. Ferulic acid was the predominate phenolic acid in Trego wheat bran produced in Colorado (USA), and accounted for 59-60 % of the total phenolic acids on a per weight basis, along with significant levels of syringic, p-hydroxybenzoic, vanillic, and coumaric acids at a concentration range of 4-33 μg/g bran (*13*). Earlier in 1992, Onyeneho and Hettiarachchy (*4*) reported that ferulic, vanillic and p-coumaric acids were major

phenolics in wheat bran extracts, along with other free phenolics including caffeic, chlorogenic, gentisic, syringic, and p-hydroxybenzoic acids. Later in 2003, Adom and others (*8*) detected ferulic acid in grain samples of 11 wheat varieties and experimental lines. Recently, Zhou and Yu (*13*) reported that ferulic acid was the major phenolic acid in bran samples of Trego wheat grown at Burlington and Walsh in Colorado (USA) and respectively accounted for 59 and 60% of the total phenolic acids, along with syringic, p-hydroxybenzoic, vanillic, and coumaric acids.

In addition to the phenolic acids, carotenoids including lutein, zeaxanthin, and β-cryptoxanthin were detected in grain samples of wheat varieties and experimental lines at concentration ranges of 25-145, 8.5-27, and 1-13.5 μg/100 g grain, respectively (*8, 14, 15*). Also noted was the presence of β-carotene in wheat (*15*). Carotenoids are considered as a group of radical scavengers through two proposed mechanisms (*16*). Carotenoid may directly react with radicals and form radical-carotenoid adducts, and/or act as electron donors to transfer an electron to radicals and form a stable carotenoidic radical cation. Carotenoids may donate an electron to phenoxyl radicals and regenerate the primary phenolic antioxidants including tocopherols. Tocopherols are another group of well-recognized natural antioxidants with potential health benefits. To date, little research has been performed to investigate the carotenoid or tocopherol profiles in wheat bran.

There are a number of established assays for antioxidant activity evaluation. Previous research has indicated that the antioxidant activity assay and the assay conditions might alter the overall antioxidant activity estimation. Our previous study of

wheat antioxidant properties showed that the relative radical scavenging capacities of Akron, Platte, and Trego wheat grains were about 17:8:1 against $DPPH^{\bullet}$ on a same weight basis, but the extract of Platte grain exhibited the strongest radical scavenging activity against $ABTS^{\bullet+}$ under the experimental conditions (*3*). In another study, we found that the order of radical $ABTS^{\bullet+}$ scavenging activities of 4 selected cereal samples, including a commercial whole-wheat cereal product, differed from that of $DPPH^{\bullet}$ scavenging capacity (*11*). These data indicated that more than one radical system should be used to evaluate antioxidant activity. The conclusion was supported by the observation by Wang and others (*17*). In their study, selected antioxidants showed different relative activities against $ABTS^{\bullet+}$ and $DPPH^{\bullet}$. This conclusion was also supported by a study conducted by Wang and Jiao (*18*). Wang and Jiao evaluated the antioxidant properties of selected berry crops using several testing systems. They observed that the berry crop that had a stronger scavenging capacity against superoxide radical did not necessarily exhibit a greater hydroxyl radical quenching activity. In addition, it was noted in our previous investigation that the testing condition or the assay design might have influence on the antioxidant activity estimation (*19*). In this study, conjugated linoleic acid (CLA) isomers, the *c*9,*t*11-CLA and *t*10,*c*12-CLA, were evaluated and compared for their DPPH radical scavenging activities using both spectrophotometric and electron spin resonance (ESR) spectrometry methods. The results showed that *c*9,*t*11-CLA had stronger radical scavenging activity when the experimental conditions were designed to test the kinetic property of the radical-antioxidant properties, whereas *t*10,*c*12-CLA would exhibit greater antioxidant capacity if the experiment was designed to evaluate the thermodynemic properties of the radical-antioxidant reaction (*19*). The results from this

study suggested the potential influence of the assay conditions on the overall antioxidant activity estimation. The previous studies of wheat antioxidants generally involved locally produced wheat varieties using different assays or testing conditions. This made it very hard to compare the data from different studies. The present study was conducted to determine the profiles of tocopherols, carotenoids, and phenolic acids in the bran fractions of the selected seven wheat varieties from four different countries. This research also examined the antioxidant properties and total phenolic contents of the bran samples. In addition, the potential correlations among individual antioxidant properties and antioxidant components were determined. This research is part of our continuous efforts to promote the improved production and utilization of value-added wheat for disease prevention and health promotion.

MATERIALS AND METHODS

Wheat bran samples. Bran samples of Swiss red, Canadian hard white, Canadian durum, Illinois soft red, and Australia general-purpose wheat were provided by the Buhler AG (Uzwil, Switzerland), whereas bran samples of wheat varieties Akron and Avalanche, represent hard winter red and white wheat, were obtained from Dr. Scott Haley in the Department of Soil and Crop Sciences, Colorado State University (Fort Collins, Colorado).

Extraction and testing sample preparation. 4 grams of each wheat bran sample was ground to fine powder using a micro-mill manufactured by Bel Art Products (Pequannock, NJ) and extracted for 15 hours with 40 mL of 50% acetone under nitrogen at ambient temperature. The 50% acetone extracts were kept in the dark under nitrogen at

room temperature until further evaluation of antioxidant properties except the $DPPH^{\bullet}$ scavenging capacity, total phenolic contents, and subjected to additional treatment for phenolic acid analyses.

Superoxide anion radical $O_2^{\bullet-}$ scavenging activity. $O_2^{\bullet-}$ scavenging activity was determined using hypoxanthine (HPX) /xanthine oxidase (XOD) system following a procedure previously described (*12*). NBT, HPX, and XOD solutions were prepared with 50 mM phosphate buffer (pH 7.4). The reaction mixture contained 0.2 mL of 0.34mM NBT, 0.7 mL of 2 mM HPX, 0.1mL of wheat antioxidant in 50% acetone, and 0.2 mL of 0.56 units/mL XOD. The decrease in absorbance was measured at 560nm every minute for a 7 min period. The $O_2^{\bullet-}$ scavenging activity was expressed as $O_2^{\bullet-}$ remaining.

Radical cation $ABTS^{\bullet+}$ scavenging activity. Radical scavenging capacity of wheat antioxidant was evaluated against $ABTS^{\bullet+}$ generated by the chemical method according to a previously reported protocol (*10, 20*). 50 μL of bran extracts was diluted with 450 μL of 50% acetone to obtain the testing samples. $ABTS^{\bullet+}$ was prepared by oxidizing 5 mM aqueous solution of ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt, with manganese dioxide at ambient temperature for 30 min. The $ABTS^{\bullet+}$-antioxidant reaction mixture contained 1.0 mL of $ABTS^{\bullet+}$ with an absorbance of 0.7 at 734 nm, and 80 μL of antioxidant testing sample or 80 μL of 50% acetone solution for the control. The absorbance at 734 nm was measured at 1 min of the reaction, and the trolox equivalent was calculated using a standard curve prepared with trolox.

ORAC assay. ORAC assay was conducted using fluorescein (FL) as the fluorescent probe according to a protocol described by Huang and others (*10, 21*). The final assay mixture contained 0.067μM of FL, 60 mM of AAPH, 300μL of wheat bran antioxidants or 50% acetone for a reagent blank. The fluorescence of an assay mixture was determined and recorded every min. The trolox equivalent was calculated using a standard curve prepared with trolox, and used to compare ORAC of wheat bran samples.

Radical DPPH scavenging activity. 5 grams of each ground wheat bran sample was extracted for 2 hours with absolute ethanol using a Soxhlet extractor, and the final volume of the extract was brought to 200 mL. Radical DPPH scavenging capacity of wheat bran extracts were determined according to the previously reported procedure using the stable 2,2-diphenyl-1-picryhydrazyl radical ($DPPH^{\bullet}$) (*3*). The initial concentration was 100 μM for $DPPH^{\bullet}$ in all antioxidant-radical reactions. The absorbance at 517 nm was measured against a blank of pure ethanol at 0, 1, 5, 10, 20, 40, 80, and 1400 min and used to estimate the remaining radical levels according to a standard curve. The kinetics of antioxidant-radical reactions was compared for wheat bran antioxidants at a final concentration of 12.5 mg bran equivalent per mL. To determine the ED_{50} value of the wheat antioxidants against DPPH radicals, seven levels of each bran extract were employed. $A_{517\ nm}$ at 80 min of reaction was used to establish the ED_{50} value under the experimental conditions. ED_{50} value is the concentration of an antioxidant to quench 50% radicals in the reaction mixture under the assay condition.

Chelating activity. 2, 2'-bipyridyl competition assay was conducted to measure the Fe^{2+} chelating activity of bran extracts (*11*). The reaction mixture contained 0.1 mL

of 1 mM $FeSO_4$ solution, 50 μL of wheat bran extract in 50 % acetone, 0.3 mL of 10 % hydroxylamine-HCl, 0.4 mL of 2,2'-bipyridyl solution (0.1 % in 0.2 M HCl), 0.8 mL of Tris-HCl buffer (pH 7.4). The absorbance at 522 nm was measured and used to determine Fe^{2+} chelating activity using EDTA as a standard.

Total phenolic contents. The total phenolic contents of wheat bran extracts were determined using Folin-Ciocalteu reagent (*3*). In brief, the reaction mixture contained 50 μL of wheat bran extracts, 3 mL of pure water, 250 μL of the Folin-Ciocalteu reagent freshly prepared in our laboratory and 0.75 mL of 20% sodium carbonate. After two hours of reaction at ambient temperature, the absorbance at 765 nm was measured and used to calculate the phenolic contents in wheat bran using gallic acid as a standard. The Folin-Ciocalteu reagent was prepared by refluxing a mixture of sodium molybdate, sodium tungstate, 85% phosphoric acid, and concentrated hydrochloric acid for 10 hours, and followed by reacting with lithium sulfateand, and oxidizing by a few drops of bromine. The resulted solution was filtered and ready for test.

Phenolic acid composition. The 50% acetone extract of each wheat bran was evaluated for the phenolic acid profile. After removing acetone, the wheat bran antioxidants were hydrolyzed with 4N NaOH, acidified using 6N HCl, and extracted with ethyl ether-ethyl acetate (1:1, v/v) according to the procedure described previously (*10, 13*). The ethyl ether-ethyl acetate was evaporated at 30° C using a nitrogen evaporator, and the solid residue was re-dissolved in methanol, filtered through a 0.45 μm membrane filter, and kept in the dark under nitrogen until high performance liquid chromatography (HPLC) analysis. Phenolic acid composition in the methanol solution was analyzed by

HPLC using a Phenomenex C18 column (250 mm × 4.6 mm) according to an established protocol (*10, 13*). Phenolic acids were separated using a linear gradient elution program with a mobile phase containing solvent A (acetic acid/H_2O, 2:98, v/v) and solvent B (acetic acid/acetonitrile/ H_2O, 2:30:68, v/v/v). The solvent gradient was programmed from 10 to 100% B in 42 min with a flow rate of 1.5 mL/min (*10, 13*). Identification of phenolic acids was accomplished by comparing the retention time of peaks in wheat bran samples to that of the standard compounds. Quantification of individual phenolic acid was conducted using total area under each peak with external standards.

Carotenoid composition. Carotenoids were extracted and analyzed using HPLC-DAD-ESI-MSMS (high performance liquid chromatography-diode-array-detector-electrospray ionization tandem mass spectrometry) method (*14, 15*). Briefly, 200 mg of the ground wheat bran sample was extracted with 10 mL of methanol/tetrahydrofuran (1:1, v/v) at ambient temperature for 15 hours and then sonicated for another 10 minutes. The resulting extraction mixture was subjected to a centrifugation at ambient temperature. After centrifugation, the supernatant was filtered through a 0.20 μm membrane filter, and kept in dark under nitrogen until HPLC analysis of carotenoids, as well as for tocopherol analysis. HPLC analysis was performed using a TSQ Quantum tandem mass spectrometry (Thermo-Finnigan, San Jose, CA, USA) equipped with an ESI interface and Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA). HPLC separation was accomplished according to a previously described protocol with modifications (*15*). The HPLC was performed using a Zorbax SB C18 column (Agilent Technologies, Palo Alto, CA, USA), 1.0 mm i.d. × 50mm, 3.5 μm particle size, at room temperature. 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 50% to 99% of solvent B and the flow rate was increased from 0.20 to 0.27 mL/min in the first 10 min, and 2) 99% of solvent B and flow rate of 0.27 mL/min for 10 min. The HPLC column was re-equilibrated for another 10 minutes with 50% of solvent B, prior to injection of the next sample. The wavelength of UV detection was set at 440 nm. The TSQ Quantum was operated in the positive-ion mode under following conditions: nitrogen (> 99.7%) was used for sheath gas and auxiliary gas at pressure of 30 psi and 5 units, respectively. The temperature of the heated capillary was maintained at 300° C, and the spray voltage of ESI was set at 4.5 kV. A collision induced dissociation (CID) was achieved using argon as the collision gas at the pressure adjusted to more than 1.0 mTorr above the normal, and the applied collision offset energy was set to – 45 eV. Identification of carotenoids was accomplished by comparing the HPLC retention time and selected reactant monitoring (SRM) analysis of the sample peaks with that of the authorized pure commercial carotenoid compounds. The m/z: from 568.6 (molecular ion) to 157.3 (major fragment) was set for lutein and zeaxanthin, and m/z: 552.6 $\rightarrow$ 145.3, 536.6 $\rightarrow$ 119.3 was set for cryptoxanthin and β-carotene, respectively. Data was acquired with Xcalibur software system (Thermo-Finnigan, San Jose, CA, USA). The quantification for each carotenoid compound was conducted using the total ion counts with an external standard.

Tocopherol composition. The methanol/tetrahydrofuran extracts of the bran samples prepared for carotenoid analysis were used to evaluate the α-, δ-, and γ-tocopherol concentrations in wheat bran samples. HPLC separation was performed using a Zorbax SB C18 column (Agilent Technologies, Palo Alto, CA, USA), 1.0 mm i.d. x

30mm, 3.5 μm particle size, at room temperature. The tocopherols were eluted using a mobile phase of water as solvent A and acetonitrile as solvent B. The gradient procedure was as follows: 1) the gradient was linear from 80% to 99% of solvent B and the flow rate was 0.3 mL, and 2) 99% of solvent B was kept for 10 min. The HPLC column was re-equilibrated for another 10 minutes with 50% of solvent B, prior to the injection of the next sample. Identification of tocopherols was conducted by comparing the HPLC retention time and selected reactant monitoring (SRM) analysis of the sample peaks with that of the authorized pure commercial tocopherol compounds. The m/z: from 430.6 (molecular ion) to 165.3 (major fragment) was set for α-tocopherol, and m/z: 416.6 → 151.3 and 402.6 → 137.3 were set for γ-tocopherol and δ-tocopherol, respectively. The quantification for each tocopherol was accomplished using the total ion counts with external standards.

Statistical analysis. Data were reported as mean ± SD for triplicate determinations. Analysis of variance and least significant difference tests (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL) were conducted to identify differences among means, while a Pearson Correlation test was conducted to determine the correlations among means. Statistical significance was declared at $P < 0.05$.

RESULTS

Superoxide anion radical ($O_2^{\bullet-}$) scavenging activity. Significant $O_2^{\bullet-}$ scavenging activity was detected in all tested bran samples, while the bran of Canadian durum wheat had the greatest capacity to react with and quench $O_2^{\bullet-}$ on a per weight basis, under the experimental conditions (Table 3.1). The bran extract of Canadian

durum wheat quenched about 10 % more anion radicals in the radical-antioxidant reaction mixture than that scavenged by Australia general-purpose wheat bran. Bran extracts of different wheat varieties might significantly differ in their $O_2^{\bullet-}$ scavenging activities (Table 3.1). Correlation was detected between $O_2^{\bullet-}$ scavenging activity and total phenolic content ($r = 0.83$ and $P = 0.02$).

Radical cation scavenging activity. The $ABTS^{\bullet+}$scavenging capacity of the bran extracts ranged from 17.5 to 19.7 μmoles trolox equivalent (TE) per gram of bran (Table 3.1). Wheat bran sample might significantly differ in their radical cation scavenging activities. The greatest $ABTS^{\bullet+}$scavenging capacity was detected in the Swiss red wheat bran, whereas the bran of Australia general-purpose wheat was least effective to directly react with and quench $ABTS^{\bullet+}$ in the reaction mixture, under the experimental condition. $ABTS^{\bullet+}$ scavenging capacity was not correlated to any other tested antioxidant activities or phytochemical levels.

ORAC assay. All bran extracts exhibited significant ORAC (Table 3.1). The greatest ORAC value of 124 μmoles TE/g was observed in the bran extract of the Canadian hard white wheat, and followed by that of Swiss red wheat. The lowest ORAC value of 45 μmoles TE/g, which was about 36% of that in the Canadian hard white wheat bran, was detected in the bran extract of Avalanche wheat collected from Colorado in the United States. No correlation between ORAC value and any of the tested antioxidant activity or phytochemical concentration was observed.

Table 3.1. Antioxidant properties of wheat bran*

	% $O_2^{\bullet-}$ Remaining	$ABTS^{\bullet+}$ (TE μmoles /g bran)	ORAC (TE μmoles/g bran)
Swiss red	59.19b ± 0.13	19.74d ± 0.17	107.53d ± 4.1
Canadian white	67.07d ± 0.08	18.59c ± 0.68	124.29e ± 6.70
Canadian durum	57.40a ± 0.15	18.46b,c ± 0.48	94.89c ± 10.90
US red	60.19c ± 0.14	17.78a,b ± 0.53	89.56c ± 4.54
Australian general	67.21d ± 0.15	17.45a ± 0.24	62.32b ± 8.94
US Akron	59.33b ± 0.26	18.99c,d ± 0.29	72.55b ± 2.05
US Avalanche	59.22b ± 0.08	18.85c ± 0.50	45.02a ± 8.32

* Swiss red, Canadian white, Canadian durum, US red, Australian general, US Akron, US Avalanche stand for Swiss red wheat bran, Canadian hard white wheat bran, Canadian durum wheat bran, soft red wheat bran from Illinois (USA), general-purpose wheat bran from Australia, Akron wheat bran from Walsh (CO, USA), Avalanche wheat bran from Burlington (CO, USA), respectively. ORAC stands for the oxygen radical absorbance capacity. TE stands for trolox equivalent. The final concentration of wheat bran antioxidant was 8.3 mg/mL in test solution for radical anion $O_2^{\bullet-}$ scavenging capacity determination. Within each column, means with the same letter are not significantly different ($P < 0.05$).

Radical DPPH scavenging activity. The ethanol extracts of wheat bran samples were analyzed and compared for their ED_{50} values against $DPPH^{\bullet}$ (Figure 3.1). ED_{50} is the required concentration of wheat bran antioxidants to scavenge 50% DPPH radicals in the reaction mixtures under the experimental conditions, with the ED_{50} value negatively

associated the DPPH$^{\bullet}$ scavenging activity. The ED_{50} values ranged from 6.1 mg bran equivalent per mL for Akron wheat bran to 12.1 mg bran equivalent per mL for Australian general-purpose wheat bran, indicating that individual wheat bran samples may significantly differ in their DPPH radical scavenging capacities. The kinetics of each wheat antioxidant-DPPH radical reaction was determined and reported in the Figure 3.2. Bran extract of Canadian durum wheat had the greatest initial rate in reacting with DPPH$^{\bullet}$, and quenched larger quantity of the radicals in the system when the antioxidant-DPPH$^{\bullet}$ reaction approached maximum scavenging capacity. Interestingly, bran extract of Swiss red wheat had a greater initial reaction rate than that of Akron wheat, but bran extract of Akron wheat quenched more radicals in the testing system when the radical-antioxidant reaction max developed (Figure 3.2). These data indicated that the bran extract from Swiss red wheat was a kinetically more active scavenger against DPPH$^{\bullet}$, whereas bran extract of Akron wheat was a stronger radical inhibitor under the testing conditions. The DPPH$^{\bullet}$ scavenging capacity was positive correlated with total phenolic contents ($r = 0.91$, $P = 0.01$).

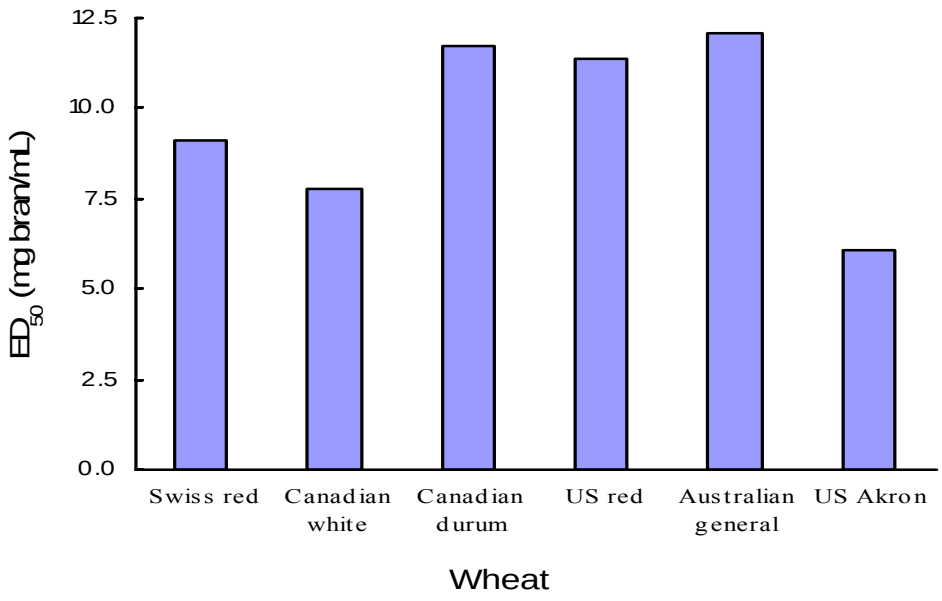

Figure 3.1. ED_{50} of wheat bran extracts against DPPH radicals.

Swiss red, Canadian white, Canadian durum, US red, Australian general, US Akron, US Avalanche stand for Swiss red wheat bran, Canadian hard white wheat bran, Canadian durum wheat bran, soft red wheat bran from Illinois (USA), bran of Australia general-purpose wheat, Akron red wheat bran from Walsh (Colorado, USA), and Avalanche wheat bran from Burlington (Colorado, USA), respectively. All tests were conducted using ethanol extracts. The initial $DPPH^{\bullet}$ concentration was 100 μM in all reaction mixtures. ED_{50} is the concentration of wheat bran extracts to quench 50% of DPPH radicals in the reaction mixture within 80 minutes under the experimental conditions.

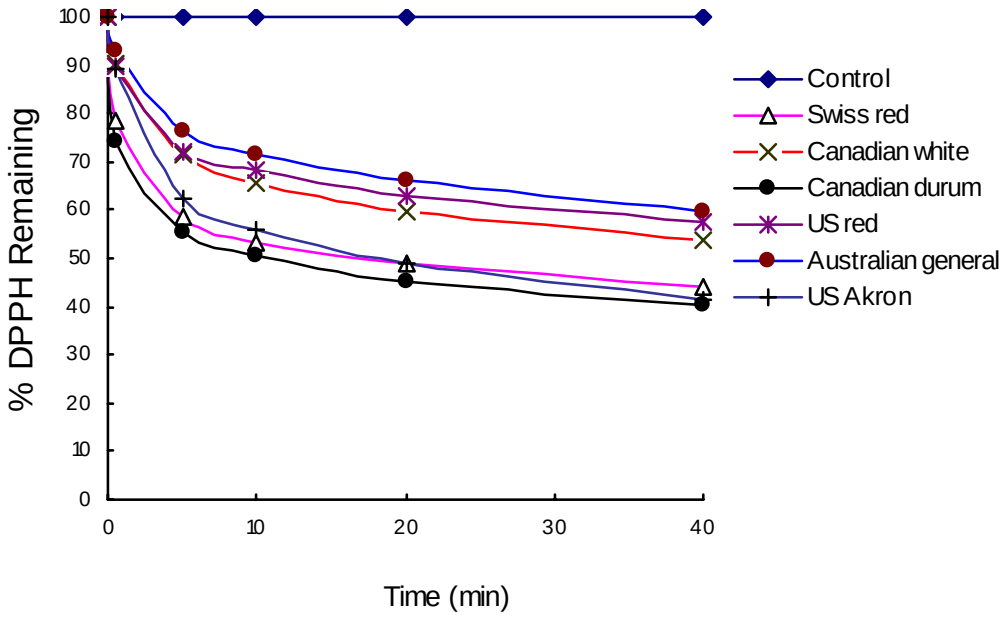

Figure 3.2. Radical DPPH scavenging activities of wheat bran extracts.

Swiss red, Canadian white, Canadian durum, US red, Australian general, US Avalanche stand for Swiss red wheat bran, Canadian hard white wheat bran, Canadian durum wheat bran, soft red wheat bran from Illinois (USA), bran of Australia general-purpose wheat, and Avalanche wheat bran from Burlington (Colorado, USA), respectively. All tests were conducted using the ethanol extracts. The initial $DPPH^{\bullet}$ radical concentration was 100 μM in all reaction mixtures, while the final concentration of wheat extracts was 12.5 mg wheat equivalent per mL.

Chelating activity. The chelating properties of the 50% acetone extracts of wheat bran samples were expressed as EDTA equivalents per gram of bran (Figure 3.3). The Fe^{+2} chelating activity of the bran samples ranged from 1-1.9 mg EDTA equivalent per gram of bran. Significant differences in chelating activity was observed among some of the wheat bran samples (Figure 3.3). The bran extract of Akron wheat from Colorado

in the United State showed the strongest chelating capacity, whereas the bran extracts of soft red wheat from Illinois (USA) had the lowest chelating activity. The chelating activity was not correlated with any tested antioxidant activity or antioxidant concentration.

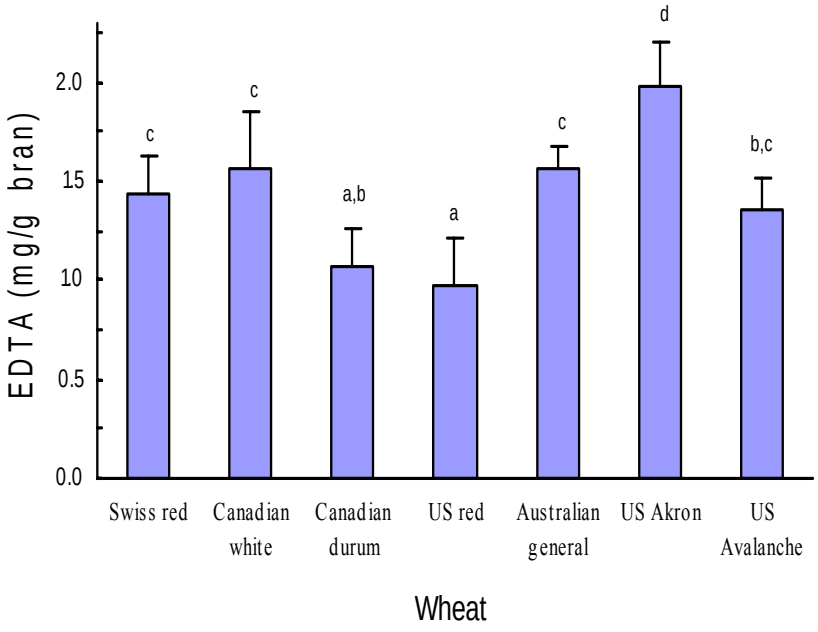

Figure 3.3. Chelating capacity of wheat bran samples.

Swiss red, Canadian white, Canadian durum, US red, Australian general, US Akron, US Avalanche stand for Swiss red wheat bran, Canadian hard white wheat bran, Canadian durum wheat bran, soft red wheat bran from Illinois (USA), bran of Australia general-purpose wheat, Akron red wheat bran from Walsh (Colorado, USA), and Avalanche wheat bran from Burlington (Colorado, USA), respectively. The vertical bars represent the standard deviation (n = 3), and values marked by the same letter are not significantly different ($P < 0.05$).

Total phenolic content. The wheat bran samples were examined and compared for their total phenolic contents (TPC) expressed as gallic acid equivalent (GE). The seven bran samples might differ from each other in their TPC (Figure 3.4). The greatest TPC of 2.9 mg GE/g bran was detected in Avalanche wheat from Colorado (USA), while bran of general-purpose wheat from Australia had the lowest TPC value of 2.2 mg GE/g bran. TPC was correlated with the $O_2^{\bullet-}$ scavenging capacity of the 50% acetone extracts ($r = 0.83$, $P = 0.02$) and the DPPH scavenging capacity of the ethanol extracts ($r = 0.91$ and $P = 0.01$).

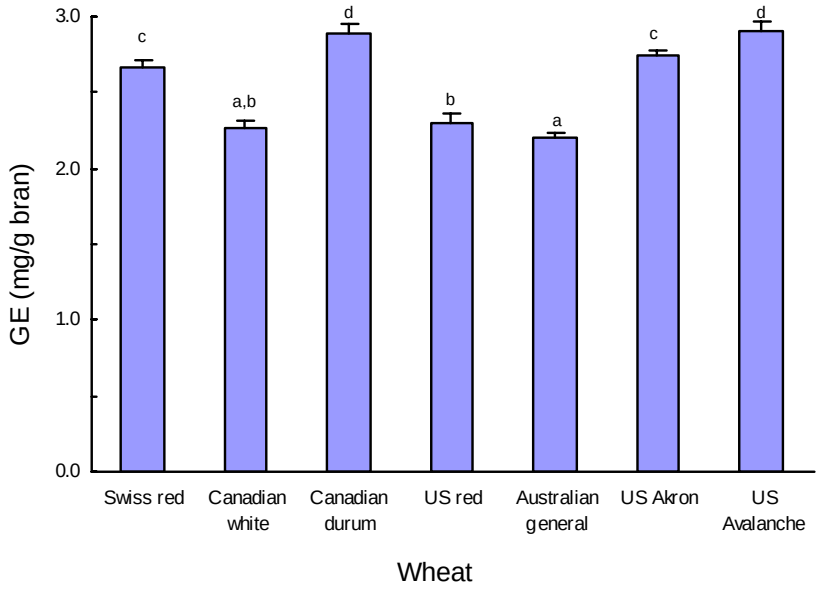

Figure 3.4. Total phenolic contents of wheat bran samples.

Swiss red, Canadian white, Canadian durum, US red, Australian general, US Akron, US Avalanche stand for Swiss red wheat bran, Canadian hard white wheat bran, Canadian durum wheat bran, soft red wheat bran from Illinois (USA), bran of Australia general-purpose wheat, Akron red wheat bran from Walsh (Colorado, USA), and Avalanche wheat bran from Burlington (Colorado, USA), respectively. The vertical bars represent

the standard deviation (n=3). Values marked by the same letter are not significantly different ($P < 0.05$).

Phenolic acid composition. Ferulic, syringic, p-OH benzoic, vanillic, and coumaric acids, were detected in all 7 tested wheat bran samples (Table 3.2). Ferulic acid was the predominant phenolic acid in all tested bran samples and accounted for about 46.1-67.2 % of the total identified phenolic acids on a per weight basis. Bran of Akron wheat (USA) had the greatest concentration of total phenolic acids (359 μg/g) and ferulic acid (230.5 μg/g). Bran samples of Swiss red and US soft red wheat also had total phenolic acids above 300 μg/g with ferulic acid level over 200 μg/g (Table 3.2).

Table 3.2. Phenolic acid composition of wheat bran*

	p-OH Benzoic (μg/g bran)	Vanillic (μg/g bran)	Syringic (μg/g bran)	Coumaric (μg/g bran)	Ferulic (μg/g bran)
Swiss red	19.65d ± 0.35	16.55b ± 0.27	57.15c ± 0.47	9.01e ± 0.02	209.3e ± 0.33
Canadian white	29.50f ± 0.03	19.31e ± 0.38	60.62d ± 0.37	8.00d ± 0.09	100.5b ± 0.54
Canadian durum	20.79e ± 0.34	26.45g ± 0.13	29.27a ± 0.36	16.21g ± 0.08	146.9c ± 0.11
US red	18.01c ± 0.33	15.01a ± 0.32	60.38d ± 0.77	10.22f ± 0.07	207.9e ± 1.19
Australian general	19.85d,e ± 0.52	17.48d ± 0.08	74.63e ± 0.16	5.49c ± 0.08	163.1d ± 0.77
US Akron	14.00b ± 1.24	25.52f ± 0.40	85.01f ± 0.44	4.38b ± 0.36	230.5f ± 1.38
US Avalanche	10.53a ± 0.10	17.11c ± 0.01	30.79b ± 0.11	3.70a ± 0.16	98.54a ± 0.39

* Swiss red, Canadian white, Canadian durum, US red, Australian general, US Akron, US Avalanche stand for Swiss red wheat bran, Canadian hard white wheat bran, Canadian durum wheat bran, soft red wheat bran from Illinois (USA), general-purpose wheat bran from Australia, Akron wheat bran from Walsh (USA), Avalanche wheat bran from Burlington (USA), respectively. p-OH Benzoic, Vanillic, Syringic, Coumaric, and

Ferulic stand for p-OH benzoic, vanillic, syringic, coumaric, and ferulic acids, respectively. Within each column, means with the same letter are not significantly different ($P < 0.05$, n = 3).

Tocopherol profile. α-, δ- and γ-tocopherols were detected in all tested wheat bran samples (Table 3.3). Total tocopherols ranged from 0.92-6.90 μmoles per 100 grams of bran. Canadian durum wheat bran contained the greatest level of total (6.90 μmoles/100g) and α-tocopherol (21.29 μg/g) among all tested wheat bran samples (Table 3.3). The highest δ-tocopherol level of 7.04 μg/g was detected in the bran of Australia general-purpose wheat, and the greatest γ-tocopherol at a level of 8.30 μg/g was observed in the US Akron wheat bran. Bran samples might significantly differ in their tocopherol compositions. Interestingly, α-tocopherol was the primary tocopherol in the bran samples of Canadian white, Canadian durum and US Akron wheat; δ-tocopherol was the major isomer in the bran samples of US soft red and Australia general-purpose wheat; and γ-tocopherol was the primary form for bran samples of Swiss red and US Avalanche wheat, suggesting that bran samples differed in both total tocopherol concentrations and the tocopherol profiles. The seven tested wheat bran samples exhibited 7.5-fold, 16.6-fold, 30.6-fold, and 3.7-fold differences in their total, α-, δ-, and γ-tocopherol contents, respectively. Significant correlation was detected between total and α-tocopherol contents (r = 0.93, $P = 0.002$). In addition, δ-tocopherol concentration was correlated with the total phenolic content (r = 0.88, $P = 0.01$), whereas γ-tocopherol level was correlated with DPPH radical scavenging capacity (r = 0.96, $P = 0.02$).

Table 3.3. Tocopherol profiles of wheat bran*

	α-Tocopherol (μg/g)	δ- Tocopherol (μg/g)	γ- Tocopherol (μg/g)	Total Tocopherols (μmoles/100g)
Swiss red	6.55c ± 0.67	5.48c ± 0.22	6.77c ± 0.38	4.51c ± 0.28
Canadian white	7.86d ± 0.95	6.41d ± 0.42	2.87a,b ± 2.11	4.10c ± 0.74
Canadian durum	21.29f ± 1.74	2.03b ± 0.39	6.06c ± 0.46	6.90e ± 0.44
US red	3.29b ± 0.10	5.96c,d ± 0.36	2.98b ± 0.12	2.96b ± 0.13
Australian general	2.29a,b ± 0.09	7.04e ± 0.39	2.23a ± 0.08	2.81b ± 0.09
US Akron	14.17e ± 1.03	0.23a ± 0.04	8.30d ± 0.45	5.34d ± 0.14
US Avalanche	1.28a ± 0.06	0.25a ± 0.05	2.32a ± 0.10	0.92a ± 0. 22

* Swiss red, Canadian white, Canadian durum, US red, Australian general, US Akron, US Avalanche stand for Swiss red wheat bran, Canadian hard white wheat bran, Canadian durum wheat bran, soft red wheat bran from Illinois (USA), general-purpose wheat bran from Australia, Akron wheat bran from Walsh (USA), Avalanche wheat bran from Burlington (USA), respectively. Within each column, means with the same letter are not significantly different ($P < 0.05$, n = 3).

Carotenoid profile. Carotenoid composition including β-carotene, zeaxanthin, lutein, and cryptoxanthin was examined for all 7 wheat bran samples (Table 3.4). Lutein and cryptoxanthin were detected in all tested bran samples. Zeaxanthin was detected in six of the seven tested bran samples, whereas β-carotene was only present in four of the tested wheat bran samples. Bran samples might significantly differ in their carotenoid profiles. The greatest total carotenoid level of 0.68 μmoles/100g bran was observed in bran of Canadian durum and Australia general-purpose wheat. Avalanche wheat bran had the lowest level of total carotenoid among all tested bran samples. Interestingly, β-cryptoxanthin was presented in all tested bran samples, but was not the major carotenoid in any individual wheat bran sample (Table 3.4). The concentration of β-cryptoxanthin was 0.18-0.64 μg/g in the tested wheat bran samples. Zeaxanthin was the primary carotenoid in three of the tested wheat bran samples, and lutein was the major carotenoid for the other four wheat bran samples including Swiss red, Canadian durum, Akron and Avalanche wheat. The greatest zeaxanthin level of 2.19 μg/g was detected in Australia general-purpose wheat bran, while the highest lutein content of 1.80 μg/g was observed in Akron wheat bran. Total carotenoid content among the seven bran samples exhibited a 5.7-fold difference, while lutein and β-cryptoxanthin concentrations had 3.6-fold and 3.5-fold differences, respectively.

Table 3.4. Carotenoids profile of wheat bran*

	β-Carotene (μg/g)	Zeaxanthin (μg/g)	Lutein (μg/g)	Cryptoxanthin (μg/g)	Total Carotenoids (μmoles/100g)
Swiss red	nd	0.48b ± 0.02	0.71b ± 0.01	0.35b ± 0.02	0.27b ± 0.01
Canadian white	0.09a ± 0.08	1.99d ± 0.06	0.77b ± 0.02	0.42b ± 0.02	0.59d ± 0.03
Canadian durum	0.40c ± 0.01	1.23b ± 0.03	1.58c ± 0.03	0.59d ± 0.01	0.68e ± 0.02
US red	nd	1.34c ± 0.02	0.74b ± 0.02	0.50c ± 0.02	0.46c± 0.01
Australian genera	0.18b ± 0.01	2.19d ± 0.03	0.79b ± 0.03	0.64d ± 0.02	0.68e ± 0.01
US Akron	0.11a ± 0.02	0.25a ± 0.01	1.80d ± 0.03	0.44b ± 0.02	0.46c ± 0.02
US Avalanche	nd	nd	0.50a ± 0.02	0.18a ± 0.01	0.12a ± 0.02

* Swiss red, Canadian white, Canadian durum, US red, Australian general, US Akron, US Avalanche stand for Swiss red wheat bran, Canadian hard white wheat bran, Canadian durum wheat bran, soft red wheat bran from Illinois (USA), general-purpose wheat bran from Australia, Akron wheat bran from Walsh (USA), Avalanche wheat bran from Burlington (USA), respectively. nd: not detected. Within each column, means with the same letter are not significantly different ($P < 0.05$, n = 3).

Antioxidant properties of bran extracts on a per unit of TPC basis. Chelating activity, radical cation $ABTS^{\bullet+}$ scavenging property, and ORAC, on a per milligram of TPC basis, were calculated and expressed as (EDTA Eq/mg of TPC), (TE Eq/mg of TPC), and (ORAC/mg of TPC) (Table 3.5; see table footnote for details). Bran of Canadian white wheat had the greatest $ABTS^{\bullet+}$ scavenging property, the strongest chelating activity against Fe^{2+}, and the highest ORAC value on a per unit of TPC basis.

The same hierarchy of (TE Eq/mg of TPC) and (ORAC/mg of TPC) was observed in the 7 wheat bran samples from four different countries, although the correlation was not significantly different from zero.

Table 3.5. Antioxidant properties of bran extracts on a per unit of TPC basis*

	EDTA Eq/mg of TPC (μg)	TE Eq/mg of TPC (μmol)	ORAC/mg of TPC (μmoles TE Eq)
Swiss red	0.54b ± 0.07	7.39c ± 0.06	40.27d ± 1.54
Canadian white	0.69c ± 0.12	8.19e ± 0.30	54.74e ± 2.95
Canadian durum	0.37a ± 0.07	6.39a ± 0.17	32.83c ± 3.77
US red	0.43a,b ± 0.10	7.74d ± 0.23	38.98d ± 1.97
Australian general	0.71c ± 0.05	7.9d,e ± 0.11	28.22b,c ± 4.05
US Akron	0.72c ± 0.08	6.94b ± 0.11	26.51b ± 0.75
US Avalanche	0.47a,b ± 0.05	6.49a ± 0.17	15.5a ± 2.86

* Swiss red, Canadian white, Canadian durum, US red, Australian general, US Akron, US Avalanche stand for Swiss red wheat bran, Canadian hard white wheat bran, Canadian durum wheat bran, soft red wheat bran from Illinois (USA), general-purpose wheat bran from Australia, Akron wheat bran from Walsh (Colorado, USA), Avalanche wheat bran from Burlington (Colorado, USA), respectively. TPC stands for total phenolic contents, while EDTA Eq stands for EDTA equivalent, a measurement of the chelating capacity. TE Eq is the trolox equivalent. TE Eq/mg of TPC is the ABTS scavenging capacity on a per unit of TPC basis. ORAC represents the oxygen radical absorbing capacity. Within each column, means with the same letter are not significantly different ($P < 0.05$, n = 3).

DISCUSSION

Growing evidence suggests that diet plays an important role in disease prevention and health promotion. Consumption of whole-grain foods has been associated with the reduced risk of several aging-related health problems including cancer and cardiovascular diseases (*22-26*). Multi-mechanisms have been proposed for the anti-carcinogenic effect of whole-grain foods. These mechanisms include but are not limited to the presence and the biological actions of digestion-resistant polysaccharides and natural antioxidants (*22*). Antioxidants may modulate cellular oxidative status and protect cellular components including DNA and enzymes from oxidative damages, and consequently enhance the cell integrity and physiological function. Whole grains are excellent dietary sources of antioxidative phenolic compounds such as ferulic acid, antioxidant vitamins including vitamin E, and carotenoids such as lutein. Whole grains consist of endosperm, germ, and bran. These grain fractions may differ in their concentrations of the natural antioxidative compounds. Previous studies showed that wheat bran is a more concentrated source of phenolic acids and total phenolics, and exhibited stronger antioxidant activities than the corresponding grain kernel (*4, 6, 10*). It has also been shown that the antioxidant properties and phenolic contents in the bran varies among wheat varieties, and might be altered by the growing conditions and the interaction between wheat genotype and growing conditions (*3, 6, 7, 10, 13*). However, limited information is available about the carotenoid and tocopherol contents in wheat bran.

In the present study, bran samples of seven wheat varieties produced in four different countries were evaluated and compared for their carotenoid content including β-carotene, zeaxanthin, lutein, cryptoxanthin, and total carotenoid concentrations. Among

the 7 tested wheat bran samples, lutein ranged from 0.5-1.8 μg per gram of bran, which equals to 50-180 μg/100 g. This range is comparable to that of about 25-145 μg per 100 grams of grain detected in the whole grains of the 11 wheat varieties (*8*). These data indicate that significant level of lutein is presented in bran fraction of wheat, but lutein may not be concentrated in the bran. This conclusion was supported by the observation by Hentschel and others (*15*) that all inner layers of the durum wheat kernel and the bran had significant amount of carotenoids measured as lutein and zeaxanthin, and bran might contain less carotenoid than the inner fractions. In the present study, we detected a significant amount of zeaxanthin (123 μg/100 g) in the Canadian durum wheat bran, along with a lutein concentration of 158 μg/100 g. This makes a total of 281 μg/100 g for lutein and zeaxanthin. This total level is greater than that of 125-220 μg/100 g detected by Hentschel and others (*15*) in the bran samples of 8 durum wheat varieties obtained from Germany and France. However, the present study detected similar amount of lutein and zeaxanthin in the Canadian durum wheat bran, whereas Hentschel and others (*15*) observed only trace level of zeaxanthin in the grain and bran samples of the 8 durum wheat varieties. Furthermore, we determined in this study that lutein was the primary carotenoid in 4 wheat bran samples, and zeaxanthin was the major carotenoid in the other 3 wheat bran samples. This was in contrast to the observation that lutein was the predominant carotenoid in wheat grain in the 11 wheat varieties (*8*) and in the 8 durum wheat grain in the 8 durum wheat varieties (*15*). This may be partially due to the fractions of wheat used in the studies, and the difference in wheat variety and growing environmental conditions. This result also suggests that total lutein and zeaxanthin may be a preferred measurement of carotenoid contents in wheat and wheat bran.

Interestingly, lutein and zeaxanthin are positional isomers, which differ from each other only in the position of one double bond (Figure 3.5). According to the chemical structures, zeaxanthin has a longer conjugated system than lutein, and is more stable than lutein. Therefore, lutein may be isomerized to zeaxanthin under certain conditions such as storage at ambient temperature or heat generated during the milling process. It has been demonstrated that lutein content rapidly decreased during seed aging or storage (*14*), but the authors did not simultaneously measure the zeaxanthin contents. In 1997, Mortensen and Skibsted (*16)* showed that both zeaxanthin and lutein could react with phenoxyl radicals, and zeaxanthin is a slightly kinetic preferred reagent and has a greater relative first-order rate constant. These data and information suggest the possibility that lutein maybe converted to zeaxanthin during bran preparation and storage, and this conversion may increase the antioxidant activity of bran carotenoid. Future research is required to test this hypothesis.

Cereal grains are important dietary sources of tocopherols (*27-29*). Among tocopherol isomers, α-tocopherol exhibits the strongest vitamin E activity, and has the greatest reactivity against singlet oxygen (*27, 29*). The δ-tocopherol has the strongest antioxidative potency among all tocopherol isomers, and followed by the γ-, β-, and α-isomers, respectively. In the present study, significant levels of α-, δ-, and γ-tocopherols were detected in the 7 wheat bran samples. The bran samples differed in their α-, δ-, γ-, and total tocopherols, suggesting the potential influence of wheat variety and growing condition on tocopherol production in wheat bran and grain. The α-tocopherol level ranged from 1.28 to 21.29 μg/g in the 7 bran samples tested in the present study, which is comparable to the level of about 16 μg/g in the wheat bran sample from Finland (*28*), 8.2

μg/g detected in wheat flour (*29*) and the level of 9.9 μg/g observed in freshly milled whole-meal wheat flour (*27*), and 9.5-10.4 μg/g in wheat meal (*28*). This range is much lower than that of 200-240 μg/g in wheat germ (*28*), or the level of 90, 180, and 150 μg/g in the olive, soybean, and peanut oils (*29*). The α-tocopherol detected in this study is the (R,R,R)-α-tocopherol, which is the natural form of α-tocopherol and is preferentially retained and distributed throughout the body, although all tocopherols are absorbed equally after ingestion (*30*). The most recent U.S. recommended daily allowance (RDA) suggests that healthy adults need 11-15 mg (R,R,R)-α-tocopherol to meet the vitamin E requirement (*31*). Wheat bran and whole-grain may significantly contribute to the daily dietary intake of vitamin E to meet life requirement, prevent deficiency symptoms in normal humans, and may prevent several chronic diseases.

It is widely accepted that the final step in α-tocopherol biosynthesis is to add a methyl group in γ-tocopherol molecule catalyzed by γ-tocopherol methyltransferase (*30*). Shintani and DellaPenna (*30*) demonstrated that over-expression of the γ-tocopherol methyltransferase in *Arabidopsis* seeds through a genomic-based approach shifted tocopherol composition in favor of α-tocopherol in the seed oil. In contrast to the previous observations, we detected significant levels of δ- and γ-tocopherols in wheat bran samples. The greatest concentration of δ-tocopherol was 7.04 μg/g in the Australia general-purpose wheat bran, and the highest γ-tocopherol at a level of 8.3 μg/g was observed in the Akron wheat bran from USA. Individual wheat bran differed in their ratios of α- and γ-tocopherols. These data suggest the potential to further increase the α-tocopherol level by modulating the γ-tocopherol methyltransferase levels through wheat

breeding or genetic modification to obtain novel wheat varieties rich in vitamin E. These data also suggest the potential to increase the level of γ-tocopherol, the tocopherol isomer with the strongest antioxidant activity, to produce wheat with stronger antioxidant capacity.

In agreement with previous observations (*4, 10, 13, 32*), ferulic acid was the predominant phenolic acid detected in the bran extracts of the 7 wheat samples and accounted for 46-70 % of the total phenolic acids on a per weight basis. Ferulic acid ranged from 99 to 231 μg per gram of bran among the 7 bran samples. This range is comparable to that of 91-111 μg/g detected in the bran extracts of Trego wheat grown at different locations (*13*). Ferulic acid has been evaluated for its antioxidant properties (*32*) and potential application as an analytical parameter in rapid determination of bran carryover in flour during milling (*33*). In addition, the bran samples of the 7 selected wheat differed significantly in their total carotenoid contents, total phenolic contents, phenolic acid compositions, and antioxidant properties, confirming the previous observation that genotype and growing conditions may influence the production of phytochemicals including antioxidants in wheat (*3, 6, 8, 13, 15*).

In conclusion, this research supports the previous observation that bran samples prepared from different wheat varieties grown under different conditions may differ in their antioxidant properties and phytochemical compositions. Wheat bran is an excellent source of dietary natural antioxidants and phenolic acids, and may contribute to total dietary carotenoids and tocopherols.

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Chapter 4. Phenolic Acid Composition and Antioxidant Properties of Maryland-Grown Soft Red Winter Wheat

ABSTRACT

Soft wheat is one of the major crops in Maryland, with little information available about the potential beneficial components in soft wheat. This study examined eight selected Maryland grown soft wheat varieties or experimental lines for their phenolic acid composition , and their antioxidant properties including Fe^{2+} chelating capacity and free radical scavenging activities against radical cation $ABTS^{\bullet+}$. The results showed that all tested soft wheat grain samples contained significant amounts of phenolic acids. Vanillic, syringic, *p*-coumaric, and ferulic acids were found in the soluble free, soluble conjugated and insoluble bound forms in the grain extracts, with ferulic acid as the predominant phenolic acid. Eight soft wheat varieties differed in their antioxidant properties. The tested wheat grain samples exhibited $ABTS^{\bullet+}$ scavenging capacity of 14.3 – 17.6 μmoles TE/g and chelating activity against Fe^{2+} of 111.1 -393.7 μg EDTA equivalent/g. These data suggest the possibility to produce soft wheat varieties rich in selected health beneficial factors for optimum human nutrition though breeding programs.

INTRODUCTION

Significant antioxidant activities have been detected in wheat and wheat-based food products (*1*-*9*). Our previous studies detected significant antioxidant activities in hard winter wheat varieties of Akron, Trego and Platte (*Triticum aestivum*) (*1*, *2*). Grain extracts prepared from the three wheat varieties showed significant free radical scavenging capacities against stable 2,2-diphenyl-1-picryhydrazyl radicals (DPPH$^{\bullet}$) and 2,2'-azino-di[3-ethylbenzthiazoline sulfonate] radical cation (ABTS$^{\bullet+}$), Fe^{2+} chelating activities, and significant levels of phenolic components (*1*, *2*). It was also noted that these varieties of wheat might significantly differ in their antioxidant activities. In addition, the three wheat grain extracts were evaluated for their capacity in inhibiting lipid oxidation in fish oils. Trego extracts had the strongest inhibitory activity against lipid peroxidation in fish oils, on a grain weight basis (*2*). These results showed the potential of developing natural antioxidants from hard winter wheat, as well as the potential influence of genotype on antioxidant properties.

A recent study in our laboratory (*9*) showed that the growing conditions, such as solar radiation and temperature stress, might influence antioxidant properties of Akron wheat, a hard red winter wheat variety (*Triticum aestivum*). It is important to identify varieties grown at particular locations which will yield the highest concentration of antioxidants and/or other beneficial factors such as carotenoids, and thereby provide the most health benefit to consumers, while improving the farm gate value of wheat. In the state of Maryland, soft wheat is a major agricultural crop with 10.6 million bushels produced in 2002 (*10*). To date, little is known about the health beneficial factors in Maryland grown soft wheat varieties. Identifying varieties growing under local

agricultural conditions with significant levels of antioxidants and other beneficial factors not only has the potential to provide health benefit to consumers, but also to promote the value-added cultivation and use of Maryland grown soft wheat rich in these factors, and thereby enhancing the agricultural economy.

The present research was conducted to evaluate the eight selected Maryland grown soft wheat varieties for their $ABTS^{\bullet+}$ radical scavenging properties, Fe^{2+} chelating capacities, and phenolic acid composition.

MATERIALS AND METHODS

Soft wheat samples. Eight soft red winter wheat genotypes, a representative sample of elite commercial varieties currently grown in Maryland, were grown in the field at Clarksville (MD) in yield trial plots at a density of approximately 350,000 plants ha^{-1}. Plots were planted following a crop of corn on October 2003. Soil type is a Chester silt loam (Fine-loamy, mixed, semiactive, mesic Typic Hapludult) with a pH = 6.7. Plots were fertilized with a fall application of 16 kg ha^{-1} of nitrogen, 40 kg ha^{-1} of phosphorus and 80 kg ha^{-1} of potassium. Additionally 56 kg ha^{-1} of nitrogen was applied in March 2004. Grain from the field plots was mechanically harvested, threshed and cleaned of debris prior to laboratory testing.

Extraction and testing sample preparation. Whole grain samples were ground to a fine powder using a micro-mill manufactured by Bel Art Products (Pequannock, NJ). Two grams of the ground grain sample were extracted with 20 mL of 50% acetone for 24 hours under nitrogen at ambient temperature, and subjected to the $ABTS^{\bullet+}$, ORAC,

chelating activity, and total phenolic acid assays. All extracts were kept in the dark under nitrogen at room temperature until further analysis.

Radical cation ABTS$^{\bullet+}$ scavenging activity. Free radical scavenging capacity of the 50% acetone extracts was evaluated against ABTS$^{\bullet+}$ generated according to previously reported protocols (*11*, *12*). Fifty microliters of wheat extracts were diluted with 450 µL of 50% acetone to create working sample solutions. ABTS$^{\bullet+}$ radicals were generated by oxidizing a 5 mM aqueous solution of ABTS with manganese dioxide under ambient temperature for 30 min. The final reaction mixture contained 1.0 mL ABTS$^{\bullet+}$ with an absorbance of 0.7 at 734 nm and 80 µL of the working sample solution or 80 µL of 50% acetone for the control. The absorbance at 734 nm was measured after a reaction time of 1 minute. Trolox equivalents per gram of wheat were calculated using a standard curve prepared with trolox.

Chelating activity. Fe^{2+} chelating activity was measured using a previously reported 2, 2'-bipyridyl competition assay (*13*). The final reaction mixture contained 500 µL of the 50% acetone extract, 30 µL of 1.8 mM $FeSO_4$ solution, 200 µL of 10% hydroxylamine-HCl, 200 µL of 1 M Tris-HCl buffer (pH 7.4), and 50 µL of 2,2'-bipyridyl solution (0.1% in 0.2 M HCl). Absorbance was measured at 522 nm to determine chelating activity using EDTA as a standard.

Phenolic acid composition. Grain samples of each soft wheat variety were analyzed for their free soluble, conjugated soluble, insoluble, and total phenolic acid compositions. The free soluble, conjugated soluble, and insoluble phenolic acids were extracted following a combined solvent and pH extraction and fractionation, and alkaline

catalyzed release of bound phenolic acids from the solid grain matrix, as shown in Figure 4.1. Acetone/methanol/water (7/7/6, v/v/v) was used to extract the free and the soluble conjugated phenolic acids, while the insoluble phenolic acids in the residue had to be released by NaOH hydrolysis before extraction (Figure 4.1). The free and conjugated phenolic acids in the acetone/methanol/water solution were separated based on their solubility under acidic condition (pH = 2). The concentration of NaOH in the hydrolysis reaction mixtures was 2 M. After evaporation of ethyl acetate and ethyl ether (1:1, v/v), each phenolic acid extract was re-dissolved in MeOH. Phenolic acid composition in the methanol solution was analyzed by HPLC using a Phenomenex C18 column (250 mm × 4.6 mm) according to an established protocol (*11, 13*). Phenolic acids were separated using a linear gradient elution program with a mobile phase containing solvent A (acetic acid/H_2O, 2:98, v/v) and solvent B (acetic acid/acetonitrile/H_2O, 2:30:68, v/v/v). The solvent gradient was programmed from 10 to 100% B in 42 min with a flow rate of 1.0 mL/min (*11, 14*). Identification of phenolic acids was accomplished by comparing the retention time of peaks in the MeOH solution to that of the standard compounds. Quantification of individual phenolic acid was conducted using total area under each peak with external standards.

Statistical analysis. Data were reported as mean ± SD for triplicate determinations. Analysis of variance and least significant difference tests (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL) were conducted to identify differences among means, while a Pearson Correlation test was conducted to determine the correlations among means. Statistical significance was declared at $P < 0.05$.

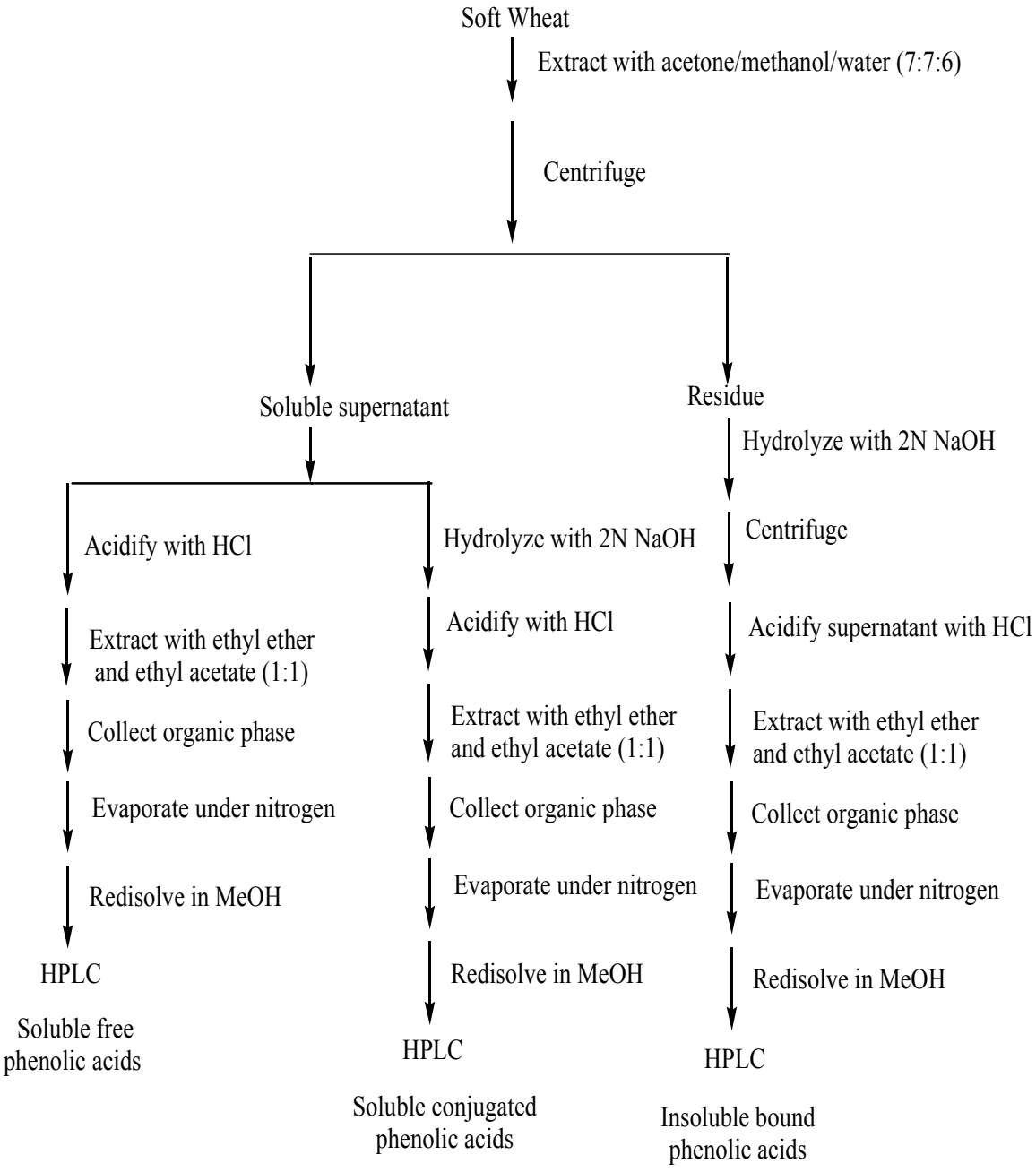

Figure 4.1. Phenolic acid extraction procedure.

RESULTS

Radical cation ABTS$^{\bullet+}$ scavenging activity. ABTS$^{\bullet+}$ scavenging activities varied from 14.3 to 17.6 μmoles trolox equivalents (TE) per gram of soft wheat grain (Figure 4.2). The greatest ABTS$^{\bullet+}$ scavenging activity was observed with the SS560 soft wheat line while the least effective one was the Vigoro Tribute variety. Also noted was that soft wheat varieties or experimental lines might significantly differ in their ABTS$^{\bullet+}$ scavenging capacities. ABTS$^{\bullet+}$ scavenging capacity was correlated with the ORAC ($r = 0.908$, $P = 0.01$).

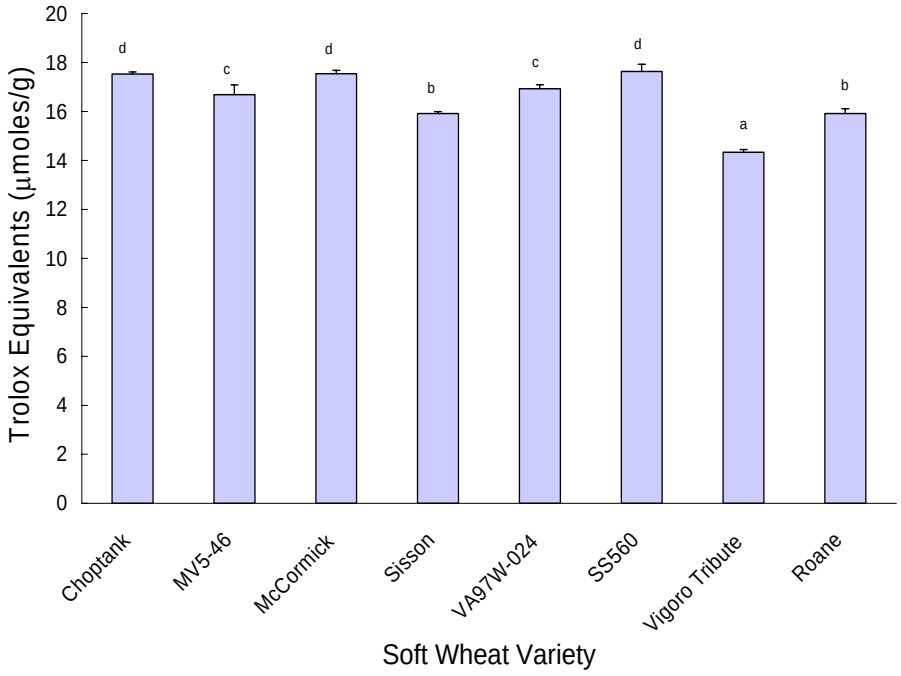

Figure 4.2. ABTS$^{\bullet+}$ radical scavenging properties of soft wheat samples. Results expressed as μmoles trolox equivalents per gram of soft wheat grains. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

Chelating activity. Fe^{+2} chelating properties of soft wheat varieties or experimental lines were expressed as EDTA equivalents per gram of soft wheat grain. Chelating activities ranged from 111.1 μg EDTA per gram grain for SS560 wheat to 393.3 μg EDTA per gram for Vigoro Tribute grain. Individual soft wheat varieties/lines might differ significantly in their Fe^{+2} chelating activities (Figure 4.3). The chelating activity was not correlated with any tested antioxidant activity or phytochemical concentration.

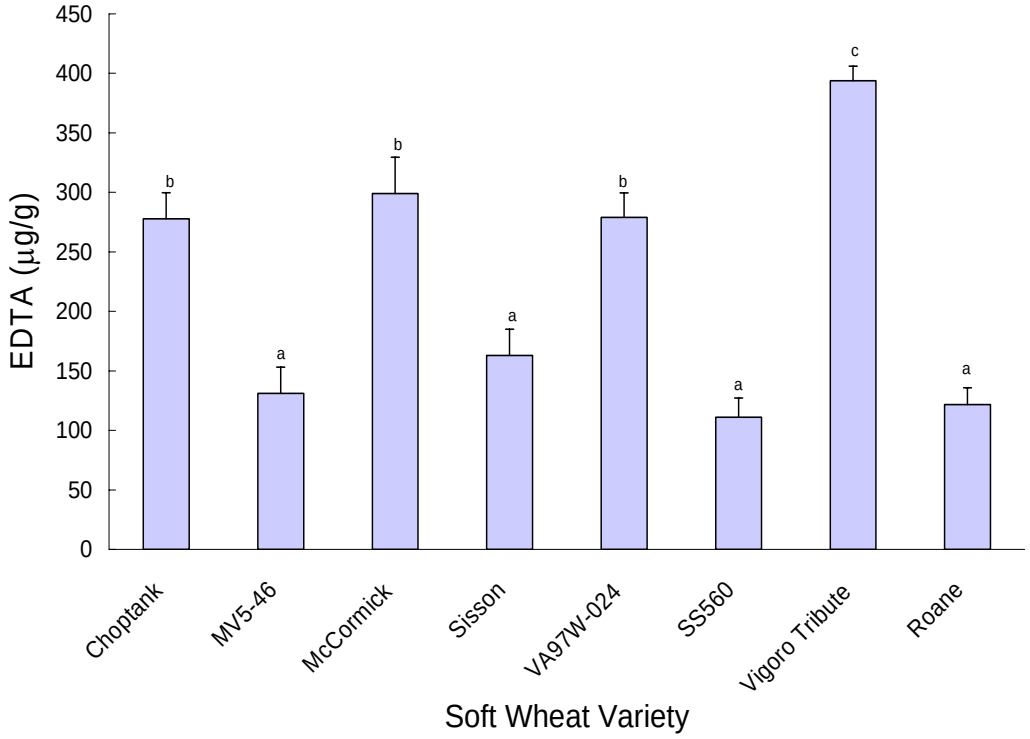

Figure 4.3. Chelating capacity of soft wheat samples.

Results expressed as μmoles EDTA per gram of soft wheat grains. All tests were conducted in triplicate, and mean values are used. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

Phenolic acid composition. Vanillic, syringic, *p*-coumaric, and ferulic acids were detected in the grain of all eight tested soft wheat samples (Table 4.1- 4.4), but no *p*-OH benzoic acid was detected in any of the tested soft wheat varieties. Grain samples of soft wheat varieties or experimental lines significantly differed in their phenolic acid compositions (Table 4.1- 4.4). Ferulic acid was the predominant phenolic acid in all the tested soft wheat varieties or lines (Tables 1a-d). Most of the ferulic acid in the soft wheat grain was insoluble bound with a concentration range of 406.7 - 587.8 μg per gram of grain (Table 4.1). This level was about 89.2-94.6% of total ferulic acid (Table 4.1) or 83.5-89.5% of the total identified phenolic acids on a per grain weight basis, respectively (Table 4.1- 4.4). Each gram of the soft wheat grain contained 0.55 - 2.31 μg soluble free and 31.95 - 47.22 μg soluble conjugated ferulic acid (Table 4.1). It was noted that the soft wheat grain had higher soluble free ferulic acid did not necessarily contain the greatest level of that in soluble conjugated or insoluble bound form (Table 4.1). For both vanillic and syringic acids, the soluble conjugated was the primary phenolic acid form (Table 4.2 and 4.3), while the insoluble bound was greatest for *p*-coumaric acid (Table 4.4). Total ferulic acid concentration on a molar basis was correlated with total phenolic acids concentration (total vanillic, syringic, p-coumaric, and ferulic acids) with correlation a coefficient (r) of 0.996 ($P = 0.01$). In addition, total soluble vanillic acid concentration was correlated with total vanillic acid concentration ($r = 0.836$, $P = 0.01$) and total soluble syringic acid concentration with total syringic acid concentration ($r = 0.989$, $P = 0.01$). Total phenolic acid concentrations were not correlated with any antioxidant activity.

Table 4.1. Ferulic acid composition of soft wheat grain samples*.

Soft wheat varieties	Soluble free (μg/g)	Soluble conjugated (μg/g)	Insoluble bound (μg/g)	Total soluble (μg/g)	Total (μg/g)
Choptank	1.69 ± 0.02b	37.02 ± 0.00d	568.4 ± 1.7e	38.71 ± 0.02c	607.1 ± 1.7e
MV5-46	1.61 ± 0.40b	33.18 ± 0.26b	524.3 ± 0.1cd	34.79 ± 0.66a	559.1 ± 0.7c
McCormick	2.01 ± 0.06bc	35.46 ± 0.29c	488.9 ± 0.1b	37.47 ± 0.23b	526.3 ± 0.3b
Sisson	1.83 ± 0.06bc	31.95 ± 0.01a	587.7 ± 0.9f	33.79 ± 0.07a	621.5 ± 1.0f
VA97W-024	1.97 ± 0.02bc	38.75 ± 0.31e	521.7 ± 0.7cd	40.72 ± 0.30d	562.4 ± 0.4c
SS560	2.31 ± 0.00c	38.38 ± 0.10e	527.1 ± 0.9d	40.69 ± 0.10d	567.8 ± 1.1d
Vigoro Tribute	2.00 ± 0.01bc	47.22 ± 0.05g	406.7 ± 1.0a	49.22 ± 0.06f	455.9 ± 1.0a
Roane	0.55 ± 0.01a	44.30 ± 0.00f	525.1 ± 0.4cd	44.85 ± 0.01e	569.9 ± 0.4d

* Results expressed as μg ferulic acid per gram of soft wheat grains. Data expressed as mean ± standard deviation (n = 2). Within each column, means with the same letter are not significantly different ($P < 0.05$).

Table 4.2. Vanillic acid composition of soft wheat grain samples*.

Soft wheat varieties	Soluble free (μg/g)	Soluble conjugated (μg/g)	Insoluble bound (μg/g)	Total soluble (μg/g)	Total (μg/g)
Choptank	0.91 ± 0.00ab	6.10 ± 0.02f	4.52 ± 0.09d	7.01 ± 0.03g	11.53 ± 0.06f
MV5-46	0.96 ± 0.03ab	4.82 ± 0.01c	3.82 ± 0.03c	5.78 ± 0.02c	9.60 ± 0.01b
McCormick	0.87 ± 0.05ab	5.64 ± 0.03e	3.56 ± 0.02b	6.51 ± 0.02e	10.07 ± 0.00d
Sisson	1.01 ± 0.06bc	5.00 ± 0.01d	5.01 ± 0.10e	6.01 ± 0.07d	11.02 ± 0.03e
VA97W-024	0.89 ± 0.00ab	4.37 ± 0.02a	4.40 ± 0.02d	5.25 ± 0.01a	9.65 ± 0.01bc
SS560	0.81 ± 0.07ab	4.70 ± 0.02b	2.93 ± 0.06a	5.51 ± 0.09b	8.44 ± 0.15a
Vigoro Tribute	1.15 ± 0.04c	5.61 ± 0.01e	3.12 ± 0.07a	6.76 ± 0.05f	9.88 ± 0.02cd
Roane	1.99 ± 0.03d	6.13 ± 0.01f	4.56 ± 0.06d	8.12 ± 0.02h	12.68 ± 0.04g

* Results expressed as μg vanillic acid per gram of soft wheat grains. Data expressed as mean ± standard deviation (n = 2). Within each column, means with the same letter are not significantly different ($P < 0.05$).

Table 4.3. Syringic acid composition of soft wheat grain samples*.

Soft wheat varieties	Soluble free (μg/g)	Soluble conjugated (μg/g)	Insoluble bound (μg/g)	Total soluble (μg/g)	Total (μg/g)
Choptank	nd	13.01 ± 0.03f	4.76 ± 0.01d	13.01 ± 0.03f	17.77 ± 0.04c
MV5-46	nd	5.79 ± 0.17a	3.07 ± 0.16ab	5.79 ± 0.17a	8.86 ± 0.01a
McCormick	nd	12.98 ± 0.06f	4.30 ± 0.39d	12.98 ± 0.06f	17.28 ± 0.33c
Sisson	nd	7.09 ± 0.01c	4.02 ± 0.02cd	7.09 ± 0.01bc	11.11 ± 0.03b
VA97W-024	nd	7.46 ± 0.09d	3.91 ± 0.09bcd	7.46 ± 0.09d	11.37 ± 0.19b
SS560	nd	8.24 ± 0.02e	2.77 ± 0.02ab	8.24 ± 0.02e	11.01 ± 0.00b
Vigoro Tribute	).60 ± 0.02a	6.37 ± 0.00b	2.70 ± 0.02ab	6.97 ± 0.02b	9.67 ± 0.00a
Roane	).85 ± 0.01b	6.47 ± 0.01b	3.39 ± 0.46abc	7.32 ± 0.00cd	10.71 ± 0.46b

* Results expressed as μg syringic acid per gram of soft wheat grains. Data expressed as mean ± standard deviation (n = 2). Within each column, means with the same letter are not significantly different ($P < 0.05$); nd, not detected.

Table 4.4. p-coumaric acid composition of soft wheat grain samples*.

Soft wheat varieties	Soluble free (μg/g)	Soluble conjugated (μg/g)	Insoluble bound (μg/g)	Total soluble (μg/g)	Total (μg/g)
Choptank	0.22 ± 0.00b	1.58 ± 0.01b	10.18 ± 0.01b	1.80 ± 0.01b	11.98 ± 0.02c
MV5-46	0.20 ± 0.05a	0.95 ± 0.23a	12.19 ± 0.02d	1.15 ± 0.28a	13.34 ± 0.26d
McCormick	nd	0.96 ± 0.01a	13.14 ± 0.25e	0.96 ± 0.01a	14.10 ± 0.24e
Sisson	0.15 ± 0.01a	1.22 ± 0.01ab	11.03 ± 0.00c	1.37 ± 0.01ab	12.40 ± 0.00c
VA97W-024	nd	0.90 ± 0.00a	9.50 ± 0.05a	0.90 ± 0.00a	10.40 ± 0.05a
SS560	0.19 ± 0.01a	1.15 ± 0.01ab	9.66 ± 0.01a	1.34 ± 0.02ab	11.00 ± 0.03ab
Vigoro Tribute	nd	1.02 ± 0.32a	10.14 ± 0.05b	1.02 ± 0.32a	11.16 ± 0.27b
Roane	0.15 ± 0.00a	1.26 ± 0.00ab	11.16 ± 0.04c	1.40 ± 0.00ab	12.56 ± 0.04c

*Results expressed as μg *p*-coumaric acid per gram of soft wheat grains. Data expressed as mean ± standard deviation (n = 2). Within each column, means with the same letter are not significantly different ($P < 0.05$); nd, not detected.

DISCUSSION

Evaluating the role of dietary antioxidants on human health, clinical evidence elucidated that consumption of whole foods such as fruits, vegetables, and whole grains and not just their known purified antioxidants have the best correlation to reduced risk of chronic diseases (*15, 16 , 17*). Whole grains in particular have been shown in 43 of 45 epidemiological studies to reduce the risk of cancer (*18*). It is hypothesized that the biological activities of natural antioxidants and other phytochemicals in addition to digestion-resistant polysaccharides in whole grains contribute to this reduced risk (19). Evaluation and demonstration of Maryland grown soft wheat varieties for their health beneficial components and antioxidant properties are the first essential step to promote the value-added production and consumption of selected soft wheat varieties rich in the desired bioactive factor(s) for prevention of chronic diseases, while enhancing the local agricultural economy.

All eight Maryland soft wheat samples displayed significant radical scavenging against $ABTS^{\bullet+}$. Scavenging activities against radical $ABTS^{\bullet+}$ cations for all soft wheat samples were comparable to that of 14.67 μmoles/g for Swiss Red wheat grain tested using the chemically generated $ABTS^{\bullet+}$ (*11*). The Fe^{2+} chelating capacities ranged from 111 to 393 μg/g for the soft wheat grains, which is comparable to that determined at about 0.37 mg/g for Swiss Red wheat grain (*11*). These data indicate that Maryland-grown soft wheat grains might not differ with previously examined hard wheat grains in their antioxidant activities, and could serve as potential dietary sources of natural antioxidants.

Interestingly, only four phenolic acids were present in all eight soft wheat varieties including vanillic, syringic, *p*-coumaric, and ferulic acids. Similar to Zhou and others' observation for Swiss Red wheat grain and fractions (*11*), ferulic acid was the predominant phenolic acid in the tested soft wheat grain samples followed by syringic and vanillic acids. However, this study did not detect 4-hydroxybenzoic acid in the tested soft wheat grains, which was detected in bran samples of hard winter wheat varieties previously (*20, 11*). Also in contrast to the findings from Zhou and other's for Swiss Red wheat grain (*11*), this study found no significant correlation between total soluble ferulic acid concentration and total concentrations of other phenolic acids for soft wheat. It was noted that the levels of total soluble ferulic, vanillic, and syringic acids in the eight soft wheat grain samples were similar to those observed in the Swiss Red wheat grain at 33.7, 4.9, and 13.7 μg/g, respectively (*11*). Comparing these and our results with those from various wheat bran samples (*20, 11*), our results also support the notion that phenolic acids are concentrated in the bran fraction of wheat.

The bran fraction of wheat includes multiple layers including most importantly aleurone. The functional purpose of the aleurone layer of bran includes a variety of protective roles against attacks by bacterial, fungal, and insect pests (*21, 22*). It also provides control of hydration during germination and is a major determinate of seed viability (*21*). Antioxidants, specifically ferulic acid and its derivatives, have long been known to be present in high concentrations in the aleurone layer of the bran fraction of wheat grain with some also present in the pericarp, nuclear envelope, and germ (*11, 21, 22*). These antioxidants are thought to contribute to the protective roles of the aleurone layer to the seed (*21*). Given that ferulic acids are found in wheat mostly associated with

aleurone layer cells which are mostly indigestible by the upper gastrointestinal tract in humans, it is believed that the colon may benefit most from these antioxidants where colonic micro flora can digest and release these antioxidants (*22*), although significant levels of phenolic acids may be released and absorbed in small intestine.

In summary, results from this study indicate significant health beneficial properties of Maryland grown soft wheat. Results from this study also suggest the possibility of production of a selected soft wheat variety rich in particular health beneficial component(s) which may benefit consumers, and consequently enhance the local agricultural economy. More research is needed to adequately know the chemical composition of the antioxidant extracts, to evaluate the effects of growing conditions on the formation of the beneficial factors, to study the influence of food formulation and processing on the availability of these factors, and to investigate their bioavailability and their potential health-promoting or disease-preventing activities in humans.

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Chapter 5. Antioxidant properties and Synergistic Effect of Wheat Phenolic Acids

ABSTRACT

Five phenolic acids commonly present in wheat grain and fractions were examined and compared for their radical scavenging properties and chelating capacities. The free radical scavenging properties were evaluated against 2,2-diphenyl-1-picryhydrazyl radical ($DPPH^{\bullet}$), radical cation ($ABTS^{\bullet+}$), peroxide radical anion ($O_2^{\bullet-}$) and hydroxyl radical ($HO^{\bullet}$), whereas the chelating properties were evaluated against Fe (II) and Cu (II) using spectrophotometric and electron spin resonance measurements. These phenolic acids differed in their properties to react with and quench $HO^{\bullet}$, $O_2^{\bullet-}$, $ABTS^{\bullet+}$, and $DPPH^{\bullet}$, as well as their capacities to form chelating complexes with transition metals. 4-Hydroxybenzoic acid had neither free radical scavenging nor chelating activity. Strong structure-activity relationships were observed in the present study. Both substituents on the phenyl ring and the conjugated carbon skeleton may influence the antioxidant properties of phenolic acids. The presence of additional methoxyl group in the ortho position of the hydroxyl group showed strong influence on the chelating property of phenolic acids and their radical scavenging capacity against $O_2^{\bullet-}$, $ABTS^{\bullet+}$, and $DPPH^{\bullet}$, but not on their $HO^{\bullet}$ scavenging activity.

INTRODUCTION

Growing evidence suggests that a high consumption of whole-grain foods, vegetables, and fruits may reduce the risk of several aging-related diseases including cancer and cardiovascular diseases (*1-7*). The beneficial effects are mainly ascribed to the phytochemicals such as insoluble and soluble fibers, vitamins, carotenoids, and natural antioxidants (*8-10*). Dietary antioxidative components may modulate cellular oxidative status and prevent important biological molecules such as DNA, protein, and membrane lipid from oxidative damage, and consequently reduce the risk of several chronic diseases including cancer and cardiovascular disease (*11-14*).

Significant levels of antioxidant activities, total phenolic contents, individual phenolic acids, carotenoids, and tocopherols were detected in wheat grain, grain fractions, and wheat-based food products, suggesting the potential utilization of wheat and wheat-based food products in human health promotion and disease prevention (*13-24*). It was noted from previous studies that both genotype and growing conditions may influence the antioxidant properties and phytochemical profiles of wheat grain and fractions (*13-18*, *21*). It was noted in a recent study of Swiss red wheat grain and fractions that phenolic antioxidants are concentrated in the aleurone fraction of wheat bran, and further post-harvesting treatment altered the availability of natural antioxidants in the aleurone (*22*). Several phenolic acids have been detected in wheat and wheat fractions, and are believed to contribute to the overall antioxidant capacities of wheat. In 1992, Onyeneho and Hettiarachchy (*15*) reported that ferulic, vanillic and 4-coumaric acids were major phenolics in wheat bran extracts, along with caffeic, chlorogenic,

gentisic, syringic, and 4-hydroxybenzoic acids. In 2003, Adom and others (*14*) detected ferulic acid in grain samples of 11 wheat varieties and experimental lines. In 2004, Zhou and Yu (*21*) reported that ferulic acid was the major phenolic acid in bran samples of Trego wheat grown at two deferent Colorado testing locations in the United States and accounted for 59-60% of the total phenolic acids, along with syringic, 4-hydroxybenzoic, vanillic, and 4-coumaric acids. In addition, a recent study showed that ferulic acid was the predominant phenolic acid in Swiss red wheat bran and consisted of 67% of the total phenolic acids on a weight basis (*22*). Significant levels of syringic, 4-hydroxybenzoic, vanillic, and 4-coumaric acids were also detected in the Swiss red wheat grain and fractions at a concentration range of 9-57.2 μg/g bran (*22*). These phenolic acids are derivatives of either benzoic or cinnamic acids (Figure 5.1). Phenolic acids are believed to contribute to the overall antioxidant activities of wheat, as well as fruits, vegetables and other grains (*14*, *21*, *22*, *25-28*).

Recently, a nutritional study was conducted to evaluate the absorption of hydroxycinnamates in humans after the administration of a high-bran wheat cereal (*28*). The results from this study suggested that ferulic and sinapic acids, both are cinnamic acid derivatives, could be absorbed by humans from wheat-based cereal foods. In addition, ferulic, vannilic, coumaric and cinnamic acids might prevent peroxidation of lipid and protein induced by hydroxyl and peroxyl radicals in synaptosomal and neuronal cell cultures (*29*). These studies suggested that wheat phenolic acids may be bioavailable and contribute to the overall *in vivo* beneficial effects of wheat and wheat-based food products.

A few studies have been conducted to investigate the antioxidant properties of the phenolic acids including the ones present in wheat and wheat bran. In 2000, Pulido and others (*30*) reported that ferulic acid had significant reducing power as measure by a modified ferric reducing/antioxidant power (FRAP) assay (*30*). In 1999, a study compared the activity of benzoic acid derivatives in prevention of human LDL oxidation to that of corresponding cinnamic acid derivatives (*31*). Ferulic, vanillic, syringic, 4-coumaric, and 4-OH benzoic acids all showed significant activity in prevention of human LDL oxidation (*31*), quenching cation radical $ABTS^{\bullet+}$ (*32*), and protecting protein molecules from radical attacks as determined by the oxygen radical absorbing capacity (ORAC) assay (*32*). Additionally, ferulic and 4-coumaric acids at a concentration of 20 μM quenched 27.3 and 7.0% of DPPH radicals in ethanol (*33*). The same study also found that ferulic acid was more effective that 4-coumaric acid in suppressing lipid oxidation in bulk methyl linoleate or in an ethanol-buffer solution of linoleic acid (*33*). To our knowledge, no investigation was conducted to evaluate these phenolic acids for their chelating capacities, radical scavenging activities against hydoxyl ($HO^{\bullet}$) and peroxide anion ($O_2^{\bullet-}$) radicals, and to test their potential synergetic effects in antioxidant properties. These data are critical for better understanding and utilization of wheat and wheat-based food products in improving human nutrition and health.

Wheat is a major agricultural commodity and dietary component across the world. The average annual farm gate value of the hard winter wheat (*Triticum aestivum*) is over $300 million in Colorado (USA) alone (*13*). Recently, wheat producers have searched for new value-added marketing opportunities for wheat because of the record low price of wheat grain. The new consumer preference of low-Carb foods may also further decrease

the global consumption of wheat. Identification of health promoting factors and novel value-added utilization of wheat is needed for enhancing its marketing potentials, and benefit both the agricultural economy and human health.

The present study was conducted to determine the radical scavenging activities of wheat phenolic acids, and their chelating capacities. Chelating capacities of phenolic acids against Fe^{2+} and Cu^{2+} were examined by spectrophotometric and electron spin resonance (ESR) spectroscopic methods, respectively. Peroxide anion ($O_2^{\bullet -}$), cation ($ABTS^{\bullet +}$), hydroxyl ($OH^{\bullet}$), and neutral DPPH radicals were employed in this study. Antioxidant-radical reactions were measured using both spectrophotometric and ESR methods. The tested phenolic acids included ferulic, 4-coumeric, vanillic, syringic, and 4-OH benzoic acids. Also determined were the potential synergetic effects among the selected phenolic acids in their antioxidant activities. In addition, the chemical structure-antioxidant activity relationships were discussed. This research is part of our continuous efforts to promote the improved production and utilization of value-added wheat in health promotion and disease prevention.

MATERIALS AND METHODS

Materials. 5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide (BMPO) was kindly provided by Dr. B. Kalyanaraman in the Biophysics Research Institute and Free Radical Research Center, at the Medical College of Wisconsin (Milwaukee, WI). High purity ferulic, 4-coumaric, syringic, vanillic, and 4-hydroxybenzoic acids were purchased from Sigma-Aldrich (St. Louis, MO). Disodium ethylenediaminetetraacetate (EDTA), hydroxylamine hydrochloride, 2,2'-bipyridyl, 2,2-diphenyl-1-picryhydrazyl radical

(DPPH·), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, hypoxanthine (HPX), xanthine oxidase (XOD), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 5,5-dimethyl N-oxide pyrroline (DMPO), diethylenetriaminepentaacetic acid (DTPA), and superoxide dismutase (SOD) were also obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents were of the highest commercial grade and used without further purification.

Superoxide anion radical $O_2^{\bullet-}$ scavenging activity. $O_2^{\bullet-}$ scavenging activity was determined by the ESR method and the xanthine/xanthine oxidase system was used to generate the $O_2^{\bullet-}$ (*34*). The antioxidant-radical reaction was initiated by addition of xanthine oxidase solution (XOD), whereas 5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide (BMPO) was used as the trapping agent (*35*). The total volume of the reaction mixture was 100 μL and the final concentrations were 2 mM for xanthine, 100 mM for BMPO, 0.2 mM for diethylenetriaminepentaacetic acid (DTPA), 2 mM for all phenolic acids, and 0.08 units/mL for XOD. All other reagents were prepared with 5 mM phosphate buffer (pH 7.4), except that phenolic acids were dissolved in 50% acetone. The ESR spectra were recorded at 2 min of reaction at ambient temperature with 10 mW incident microwave energy and 100 kHz field modulation of 1 G. Superoxide dismutase (SOD) was used as the antioxidant standard for quantification of the $O_2^{\bullet-}$ scavenging activity.

Hydroxyl radical ($HO^{\bullet}$) scavenging activity. Hydroxyl radical ($HO^{\bullet}$) scavenging capacities of the phenolic acids were examined by the ESR method. ESR assay was based on the competition between the trapping agent and the antioxidative

phenolic acids (*36*). $HO^{\bullet}$ was generated by Fenton reaction, while 5,5-dimethyl N-oxide pyrroline (DMPO) was used as the trapping agent. The reaction mixture contained 10 μL of 3 mM freshly prepared $FeSO_4$, 80 μL of 0.75 mM EDTA, 15 μL of 1 M DMPO, 15 μL of 0.5 mM H_2O_2, and 30 μL of phenolic acid solution or solvent for the blank. The final concentration was 2 mM for all phenolic acids. The ESR measurements were conducted at 1 and 20 min of each reaction at ambient temperature using a Varian E-109X-Band ESR spectrometer (Varian, Inc., Palo Alto, CA) in the Center for Food Safety and Applied Nutrition at the Food and Drug Administration (College Park, MD), with the following spectrometer settings: microwave power of 10 mW, field modulation frequency of 100 kHz, and a modulation amplitude of 1 G.

Radical cation $ABTS^{\bullet+}$ scavenging activity. Radical scavenging capacity of phenolic acids was evaluated against $ABTS^{\bullet+}$ generated by the chemical method according to a previously reported spectrophotometric procedure (*21, 22, 37*). $ABTS^{\bullet+}$ was prepared by oxidizing 5 mM aqueous solution of ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, with manganese dioxide at ambient temperature for 30 min. The $ABTS^{\bullet+}$-antioxidant reaction mixture contained 1.0 mL of $ABTS^{\bullet+}$ with an absorbance of 0.8 at 734 nm, and 100 μL of phenolic acid or 100 μL of 50% acetone solution for the control. The absorbance at 734 nm was measured at 1 min of the reaction, and the trolox equivalent was calculated using a standard curve prepared using trolox. The $ABTS^{\bullet+}$ scavenging capacity of phenolic acids was expressed as mmoles of trolox equivalent per mmole of phenolic acid.

To test the potential synergetic effect among the wheat phenolic acids against $ABTS^{\bullet+}$, total radical scavenging capacities of selected combinations of two phenolic acids were determined according to the protocol described above using $ABTS^{\bullet+}$. For instance, to determine the potential synergetic effect between ferulic and syringic acids, the selected phenolic acid combinations include (a) 100% ferulic acid, (b) 50% ferulic and 50% syringic acids, and (c) 100% syringic acids. The trolox equivalents were calculated and used to compare the total $ABTS^{\bullet+}$ scavenging capacities of the phenolic acid combinations with that of the two individual phenolic acids to determine the synergetic effect between them.

Radical DPPH scavenging activity. Radical DPPH scavenging capacities of individual wheat phenolic acids were determined by electron spin resonance (ESR) spectrometry method using the stable 2,2-diphenyl-1-picryhydrazyl radical ($DPPH^{\bullet}$) (*13*). ESR analysis was conducted using a Varian E-109X-Band ESR spectrometer (Varian, Inc. Palo Alto, CA) at ambient temperature in the Center for Food Safety and Applied Nutrition at the Food and Drug Administration (College Park, MD). Each phenolic acid solution was mixed with $DPPH^{\bullet}$ stock solution to initiate the antioxidant-radical reaction. The final concentration was 250 μM for $DPPH^{\bullet}$ in all reaction mixtures. The final concentration was 50 μM in the reaction mixture for all phenolic acids, and the control reaction contained no antioxidant. Both $DPPH^{\bullet}$ and phenolic acid solutions were prepared with 50% acetone in water (v/v). ESR signals were recorded at 1, 25, and 75 min following the start of the reaction, with a modulation frequency of 100 kHz, a sweep width of 2 G, and 20 mW incident microwave power (*13*, *38*). The scavenging activity of

each phenolic acid was estimated by comparing the $DPPH^{\bullet}$ in the antioxidant-radical reaction mixture and that in the control reaction for the same period of reaction time, and expressed as % $DPPH^{\bullet}$ remaining.

The potential synergetic effects among the wheat phenolic acids in quenching $DPPH^{\bullet}$ were examined using a spectrophotometric method. Total free radical scavenging capacities of selected combinations of the wheat phenolic acids were estimated and compared using $DPPH^{\bullet}$ (*39*). For instance, to determine the potential synergetic effect between ferulic and syringic acids, the selected phenolic acid combinations include (a) 100% ferulic acid, (b) 75% ferulic and 25% syringic acids, (c) 50% ferulic and 50% syringic acids, (d) 25% ferulic and 75% syringic acids, and (e) 100% syringic acids. The initial concentrations were 100 μM for $DPPH^{\bullet}$ and 20 μM for total phenolic acids in all antioxidant-radical reactions. Both $DPPH^{\bullet}$ and individual phenolic acid solutions were prepared with 50% acetone in water (v/v). The absorbance at 517 nm was measured against a blank of the solvent at 0.5, 1, 1.5, 2, 2.5, and 3 min of the radical-antioxidant reaction and used to estimate the remaining radical levels according to a standard curve. $A_{517\ nm}$ at 3 min of reaction was used to compare the $DPPH^{\bullet}$ scavenging capacity of each combination of phenolic acids.

To test the effect of solvents on $DPPH^{\bullet}$ scavenging capacity estimation, $DPPH^{\bullet}$-antioxidant reactions were carried out in 100% ethanol or 50% acetone under the same conditions. For instance, for the reactions carried out in 100% ethanol, both $DPPH^{\bullet}$ and phenolic acid solutions were prepared with 100% ethanol. The initial concentrations were 100 μM for $DPPH^{\bullet}$ and 20 μM for individual phenolic acids in all antioxidant-

radical reactions. $A_{517\ nm}$ at 30 min of reaction was used to compare the $DPPH^{\bullet}$ scavenging capacity of each $DPPH^{\bullet}$-phenolic acid reaction in ethanol or in 50% acetone.

Chelating activity against Fe^{2+} and Cu^{2+}. 2, 2'-bipyridyl competition assay was conducted to measure the Fe^{2+} chelating activity of individual phenolic acid and their potential synergetic effects (*23*). The reaction mixture contained 0.1 mL of 1 mM $FeSO_4$ solution, 50 μL of phenolic acid in 50% acetone, 0.3 mL of 10% hydroxylamine-HCl, 0.4 mL of 2,2'-bipyridyl solution (0.1% in 0.2 M HCl), 0.8 mL of Tris-HCl buffer (pH 7.4). The absorbance at 522 nm was measured and used to determine Fe^{2+} chelating activity with EDTA as a standard.

ESR measurements were carried out to determine the potential chelating capacity of the phenolic acids against Cu^{2+} based on a previously described condition with slight modification (*40*). Briefly, 150 μL of 10 mM individual phenolic acid solution was mixed with 150 μL of copper chloride ($CuCl_2$) solution. ESR spectrum was recorded at 1 min of reaction using the Varian E-109X-Band ESR spectrometer at ambient temperature with following instrumental settings: 20 MW incident microwave energy and 100 kHz field modulation of 2G.

Statistical analysis. Data were reported as mean ± SD for triplicate determinations. Analysis of variance and least significant difference tests (SPSS for Windows, version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL) were conducted to identify differences among means. Statistical significance was declared at $P < 0.05$.

RESULTS

Superoxide anion radical ($O_2^{\bullet-}$) scavenging activity. $O_2^{\bullet-}$ scavenging activity of the phenolic acids were evaluated by ESR measurements. These phenolic acids included 4-coumaric, ferulic, syringic, vanillic, and 4-OH benzoic acids. Ferulic and 4-coumaric acids are cinnamic acid derivatives, whereas syringic, vanillic, and 4-OH benzoic acids are benzoic acid derivatives (Figure 5.1). ESR measurements showed that syringic acid, the 3,5-dimethoxyl derivative of 4-OH benzoic acid, had the strongest $O_2^{\bullet-}$ scavenging activity among all tested phenolic acids, and followed by ferulic and 4-coumaric acids (Figure 5.2), indicating that additional *o*-methoxyl (OCH_3) group on phenyl ring may enhance the $O_2^{\bullet-}$ scavenging activity of either benzoic or cinnamic acid derivatives. Also noted was that 4-OH benzoic acid was not able to scavenge $O_2^{\bullet-}$ in the reaction mixture. The $O_2^{\bullet-}$ scavenging activities of individual phenolic acids were also compared by ESR quantification (Figure 5.3). Ferulic, syringic, 4-coumaric, and vanillic acids had $O_2^{\bullet-}$ scavenging activity, but 4-OH benzoic acid did not quench free radicals at all. Furthermore, syringic, ferulic, 4-coumaric, and vanillic acids significantly differed in their $O_2^{\bullet-}$ scavenging activities (Figure 5.3).

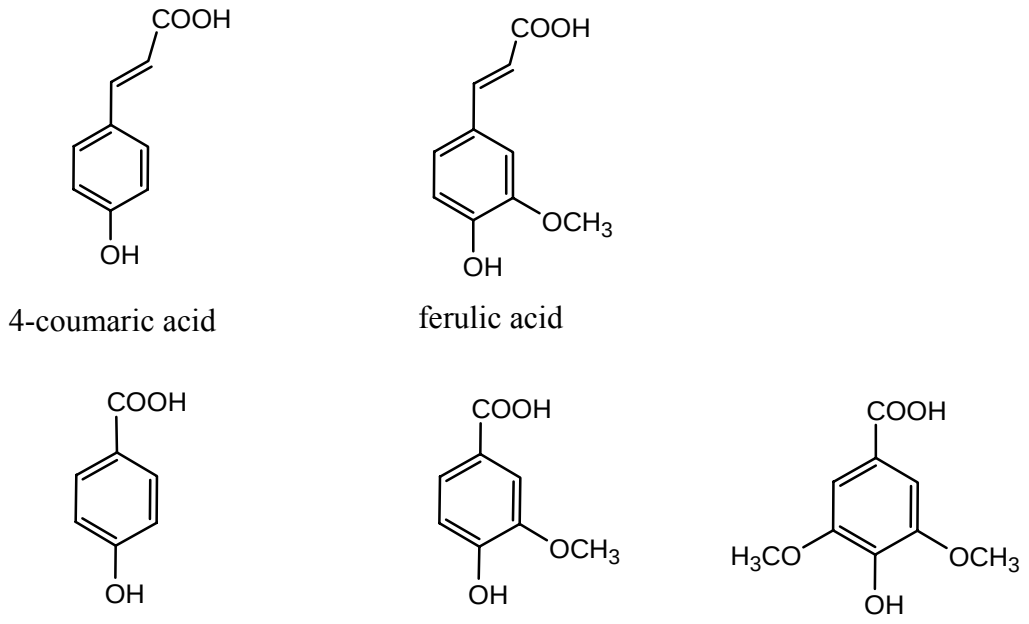


Figure 5.1. Structure of phenolic acids present in wheat grain and fractions.

4-coumaric and ferulic acids are cinnamic acid derivatives, while 4-OH benzoic, vanillic, and syringic acids are benzoic acid derivatives.

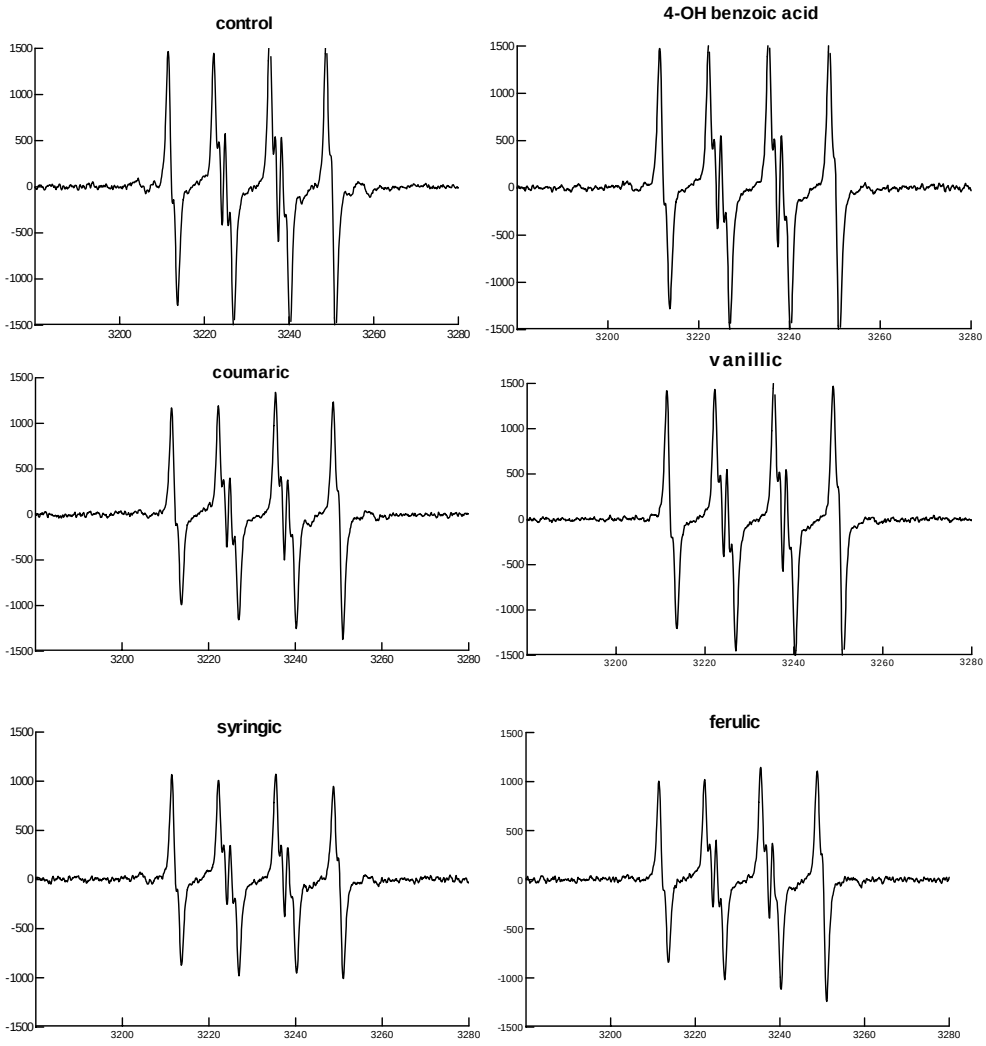


Figure 5.2. $O_2^{\bullet-}$ scavenging activities of phenolic acids determined by ESR.

Coumaric, ferulic, syringic, and vanillic stand for 4-coumaric, ferulic, syringic, and vanillic acids, respectively, while the control represents the control reaction containing no antioxidant. 4-OH benzoic acid had no $O_2^{\bullet-}$ scavenging activity. The reaction mixture contained 2 mM xanthine, 100 mM BMPO, 0.2 mM Diethylenetriaminepentaacetic acid (DTPA), 2 mM phenolic acid, and 0.08 units/mL XOD in a total volume of 100 μL. The ESR spectra were recorded at 2 min of the reaction at ambient temperature.

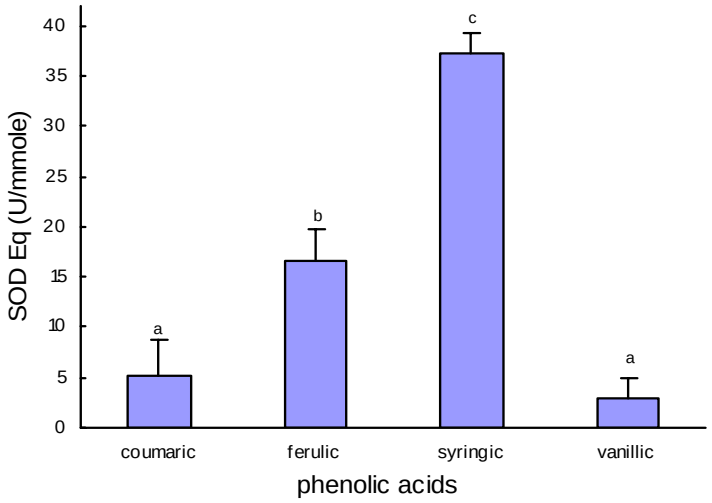
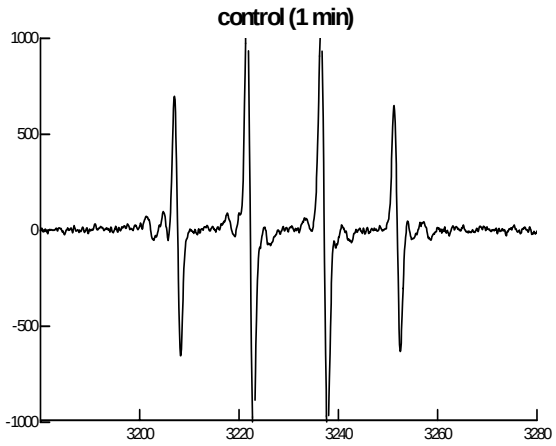


Figure 5.3. Comparison of $O_2^{\bullet-}$ scavenging capacities of the phenolic acids.

$O_2^{\bullet-}$ scavenging capacities of phenolic acids were expressed as superoxide dismutase (SOD) equivalent (U) per mmole phenolic acid). Coumaric, ferulic, syringic, and vanillic stand for 4-coumaric, ferulic, syringic, and vanillic acids, respectively. Values marked by the same latter are not significantly different ($P < 0.05$).

Hydroxyl radical (HO·) scavenging activity. 4-Coumaric, ferulic, syringic, and vanillic acids exhibited significant HO· scavenging capacities (Figure 5.4), but 4-hydroxybenzoic acid had no activity to react with and quench HO·. 4-Coumaric acid, the 4-OH derivative of cinnamic acid, showed the strongest activity in reacting with and quenching HO· (Figure 5.4). Also noted was that the HO· scavenging activities of the phenolic acids were time dependent, with a greater HO· scavenging activity associated with a longer reaction time between phenolic acid-HO· (Figure 5.4).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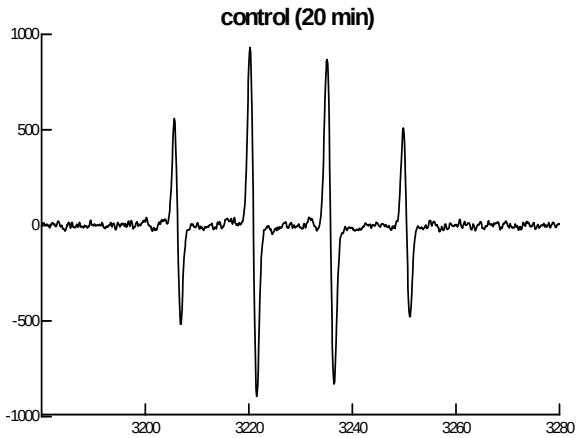
control (20 min)
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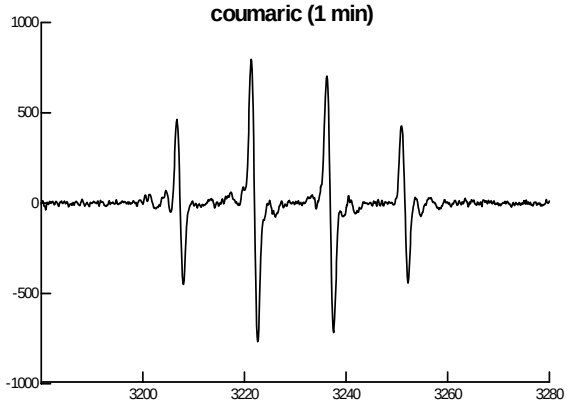
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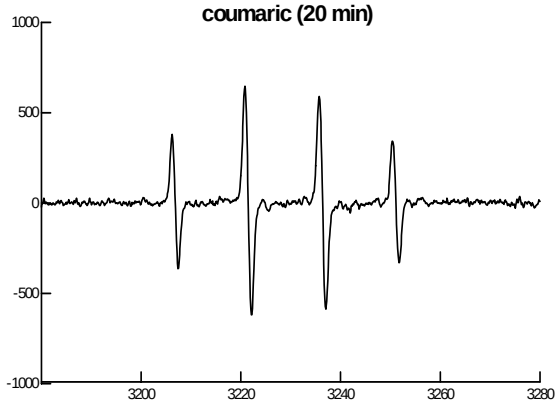
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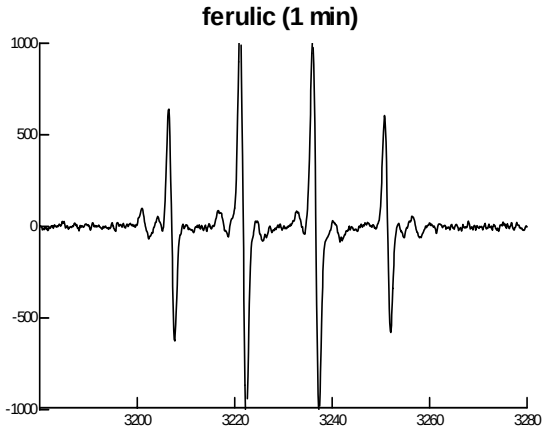
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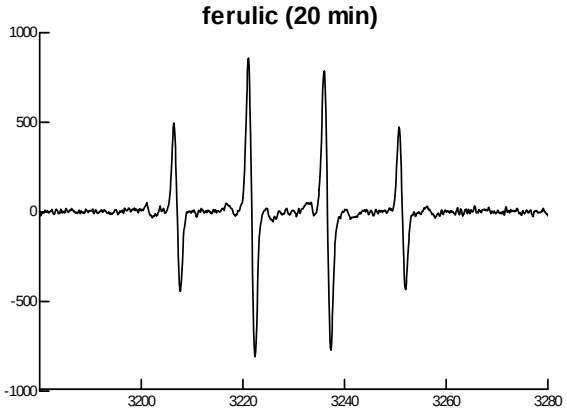
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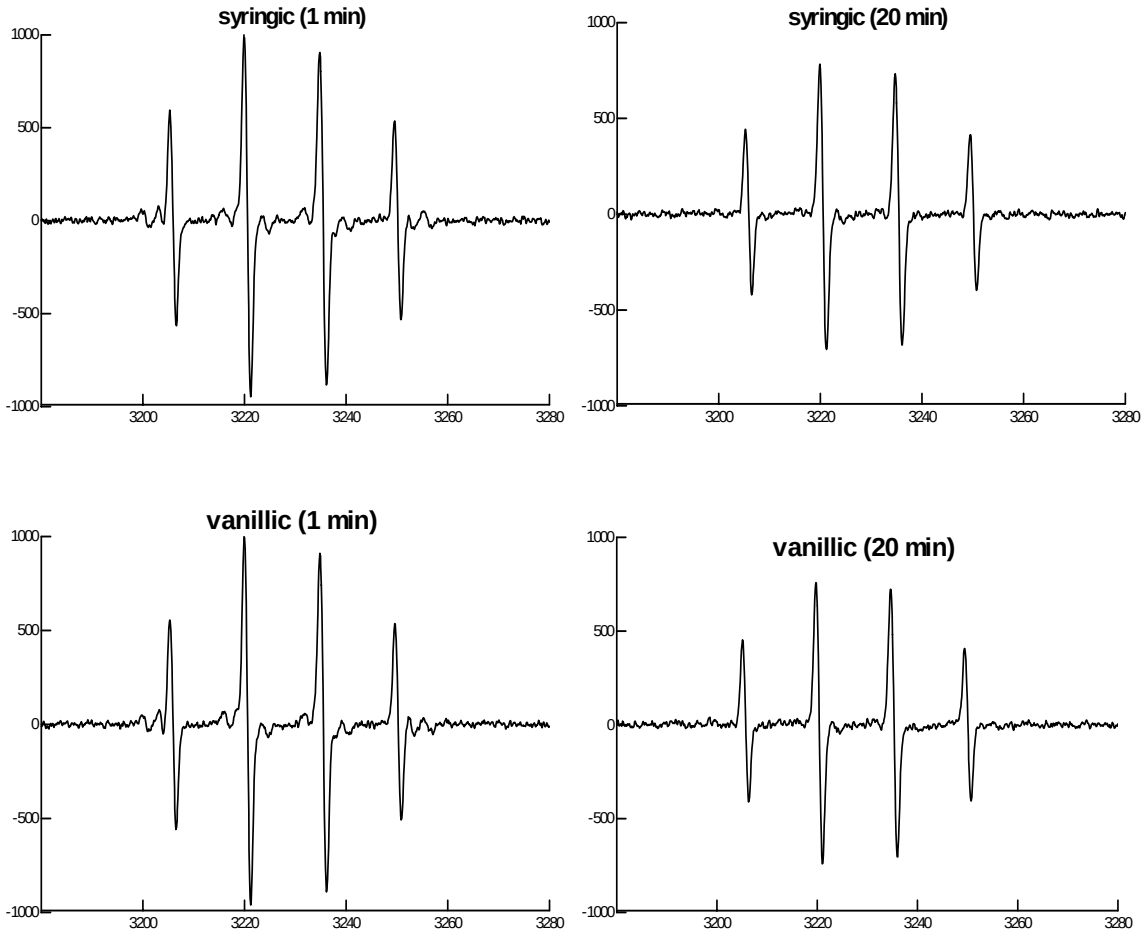


Figure 5.4. Hydroxyl radical scavenging activities of phenolic acids determined by ESR.

Coumaric, ferulic, syringic, and vanillic stand for 4-coumaric, ferulic, syringic, and vanillic acids, respectively, while the control represents the control reaction containing no antioxidant. 4-OH benzoic acid had no $HO^{\bullet}$ scavenging activity under the experimental conditions. Each reaction mixture contained 10 µL of freshly prepared 3 mM $FeSO_4$, 80 µL of 0.75 mM EDTA, 15 µL of 1 M DMPO, 15 µL of 0.5 mM H_2O_2, and 30 µL of phenolic acid solution. The final concentration of phenolic acid was 2 mM in all reaction mixtures. ESR signals were recorded at 1 and 20 min of the reaction at ambient temperature.

Radical cation ABTS$^{\bullet+}$ scavenging activity. The ABTS$^{\bullet+}$scavenging capacities of each selected phenolic acid and combinations of the phenolic acids were examined. The trolox equivalent against the ABTS$^{\bullet+}$radical ranged 1.12-1.66 mmoles per mmole of phenolic acid for ferulic, 4-coumaric, syringic and vanillic acids (Figure 5.5), but no ABTS$^{\bullet+}$scavenging capacity was detected for 4-OH benzoic acid under the experimental conditions. The four phenolic acids differed in their ABTS$^{\bullet+}$scavenging capacities. For the two cinnamic acid derivatives, ferulic acid had a greater ABTS$^{\bullet+}$scavenging capacity than that of 4-coumaric; and for benzoic acid derivatives, syringic acid had the strongest ABTS$^{\bullet+}$scavenging capacity followed by that of vanillic acid (Figure 5.5), suggesting that the presence of OCH_3 in the ortho position of the OH group may significantly enhance the ABTS$^{\bullet+}$scavenging capacity of phenolic acids. None of the phenolic acid combinations exhibited an ABTS$^{\bullet+}$scavenging capacity greater than that of each constituent phenolic acids alone (Figure 5.5), indicating no synergetic effects between these phenolic acids in their reactions with ABTS$^{\bullet+}$.

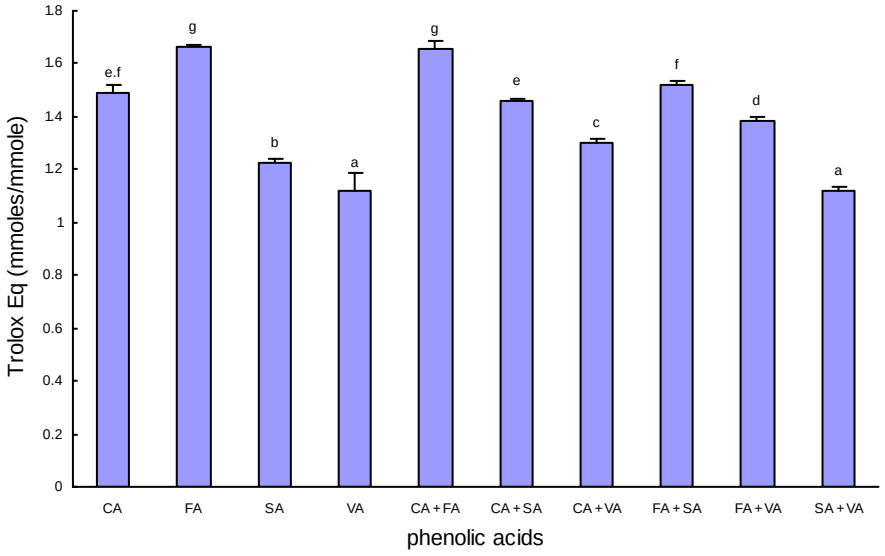
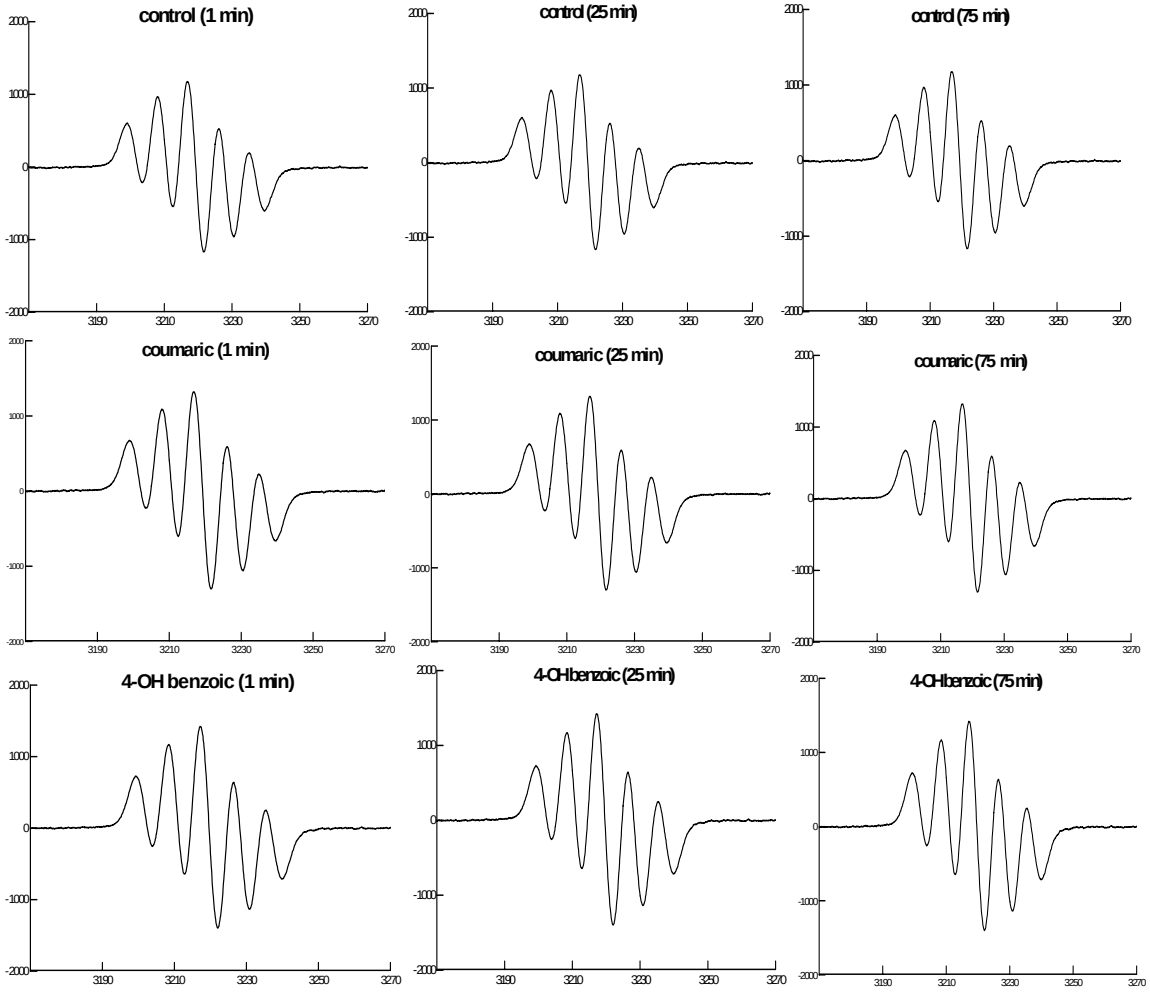


Figure 5.5. $ABTS^{\bullet+}$ scavenging properties of phenolic acids.

The $ABTS^{\bullet+}$ scavenging activities of phenolic acids were expressed as mmoles of trolox equivalent (Trolox Eq) per mmole of total phenolic acids. CA, FA, SA, VA, represent 4-coumaric, ferulic, syringic, and vanillic acid, respectively. 4-OH benzoic acid showed no $ABTS^{\bullet+}$ scavenging activity. The final concentration was 9.1 μM for all phenolic acids. The combined phenolic acids contained 50 % of each on a per mole basis. 50 % acetone was used in all reactions as the solvent. The vertical bars represent the standard deviation (n = 3), and values marked by the same latter are not significantly different ($P < 0.05$).

Radical DPPH scavenging activity. ESR measurements were conducted in 50% acetone. ESR spectra showed that derivatives of both cinnamic and benzoic acids might directly react with and quench $DPPH^{\bullet}$ in the reaction mixture (Figure 5.6). Syringic acid, the 3,5-dimethoxyl derivative of 4-OH benzoic acid, might have the strongest $DPPH^{\bullet}$ scavenging capacity followed by that of ferulic acid, the 3-methoxyl derivative of 4-OH

cinnamic acid (Figure 5.6), while 4-coumaric acid exhibited weak activity against DPPH$^{\bullet}$ (Figure 5.7). Vanillic and 4-OH benzoic acids showed no activity in quenching DPPH$^{\bullet}$ under the ESR measurement conditions (Figure 5.6 and 5.7). ESR measurements also indicated the time dependence of phenolic acid-DPPH$^{\bullet}$ reactions for both syringic and ferulic acids (Figure 5.6 and 5.7).



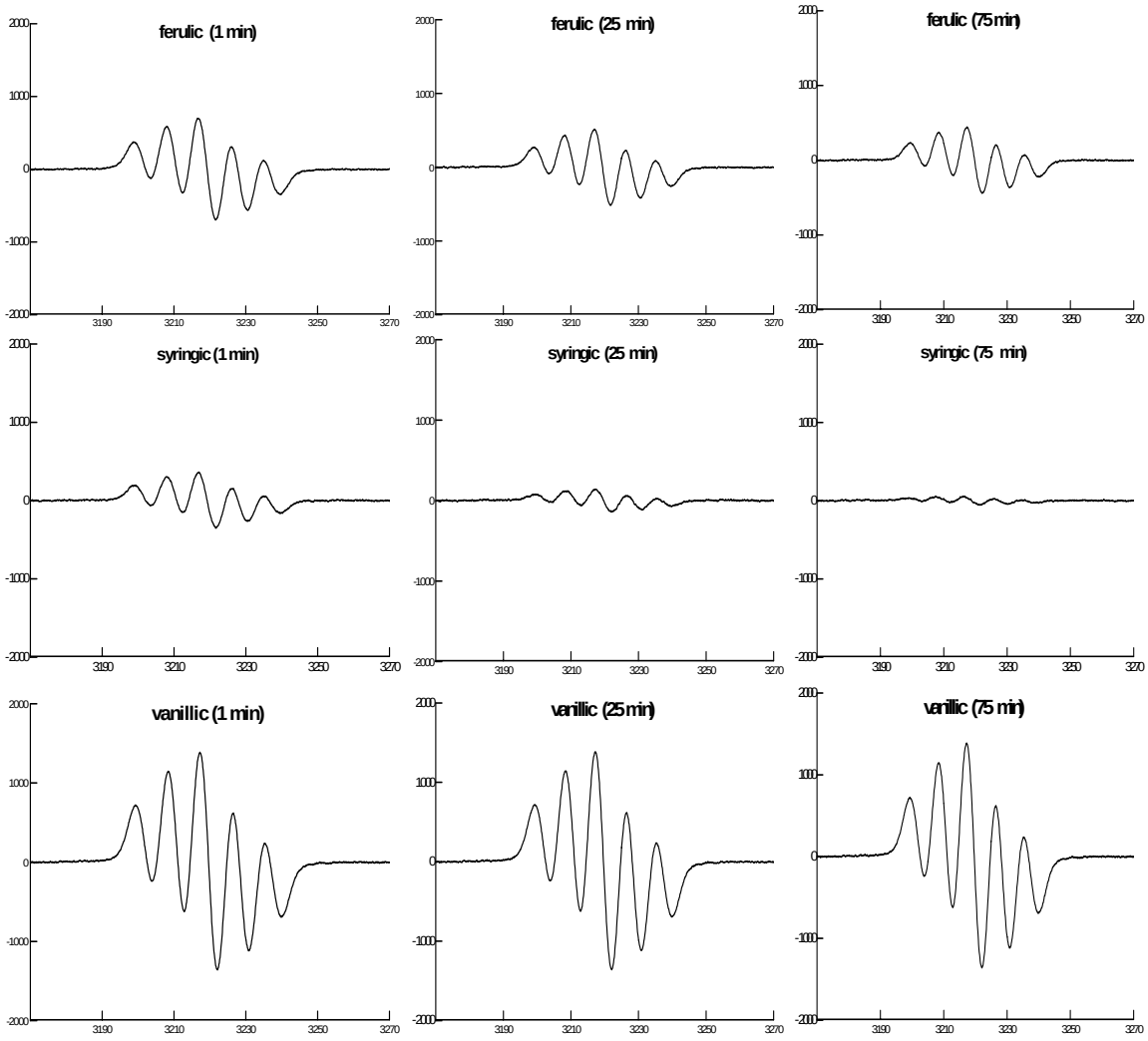


Figure 5.6. DPPH• scavenging activities of phenolic acids determined by ESR.

Coumaric, ferulic, 4-OH benzoic, syringic, and vanillic stand for 4-coumaric, ferulic, 4-OH benzoic, syringic, and vanillic acids, respectively, while the control represents the control reaction containing no antioxidant. c acid showed no DPPH• scavenging activity under the experimental conditions. The final concentrations were 250 μM for DPPH• and 50 μM for phenolic acid in all reaction mixtures. ESR signals were recorded at 1, 25, and 75 min of each reaction at ambient temperature.

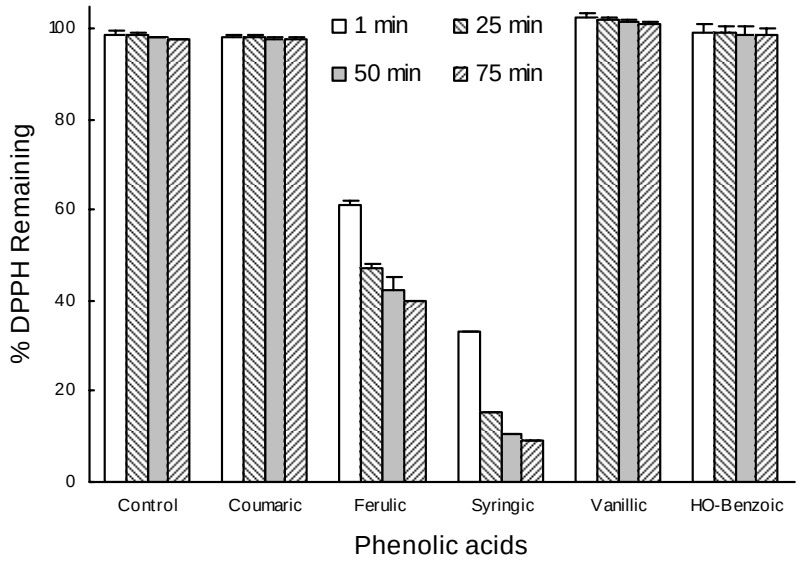


Figure 5.7. DPPH• scavenging activities of phenolic acids at different reaction times. Coumaric, ferulic, syringic, vanillic, and HO-benzoic stand for 4-coumaric, ferulic, syringic, vanillic, and 4-OH benzoic acids, respectively, while the control represents the control reaction containing no antioxidant. The DPPH• scavenging activities of phenolic acids were expressed as % DPPH• remaining. The vertical bars represent the standard deviation of each data point (n = 3).

The potential synergic effects among phenolic acids against DPPH• were examined using a spectrophotometric method with 50% acetone as the solvent for all reaction mixtures. No synergetic effect was observed between any two tested phenolic acids on the same total phenolic acid molarity basis. As an example, the testing results of the potential synergetic effect between syringic and ferulic acids in directly reacting with and quenching DPPH• were reported in Figure 5.8. None of the combinations showed greater radical scavenging capacity than that of syringic acid under the same

experimental conditions, indicating no synergetic effect between ferulic and syringic acids against DPPH radicals.

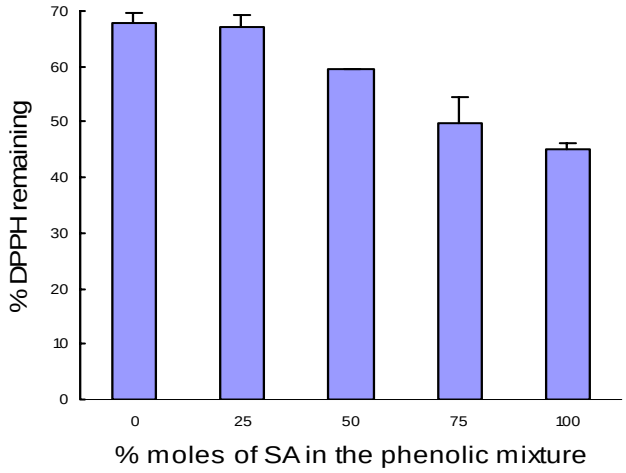


Figure 5.8. Determination of potential synergetic effects between ferulic and syringic acids against DPPH radicals.

The final concentrations were 20 μM and 100 μM for total phenolic acids and $DPPH^{\bullet}$, respectively. 0, 25, 50,75, 100 represent reaction mixtures containing 100% ferulic acid, 25% syringic and 75% ferulic acids, 50% syringic and 50% ferulic acids, 75% syringic and 25% ferulic acids, and 100% syringic acid, respectively. The vertical bars represent the standard deviation of each data point (n = 3).

Chelating activity against Fe^{2+} or Cu^{2+}. No chelating activity against Fe^{2+} was detected using a spectrophotometric method. Interestingly, vanillic and ferulic acids showed Cu^{2+} chelating capacity according to the ESR measurements, while syringic, 4-coumaric, and 4-OH benzoic acids had no interaction with Cu^{2+} (Figure 5.9).

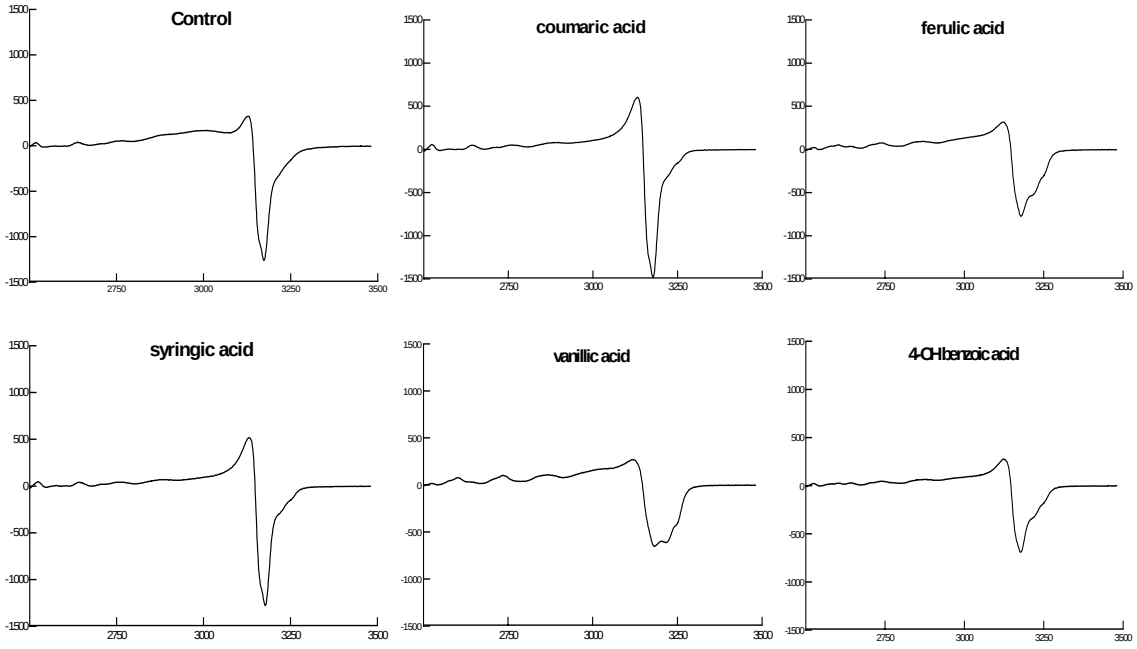


Figure 5.9. Interaction between Cu^{2+} and individual phenolic acids measured by ESR.

The final concentrations were 5 mM for each phenolic acid and 1 mM for copper chloride ($CuCl_2$). The ESR spectrum was recorded at 1 min of reaction at ambient temperature.

DISCUSSION

Significant levels of ferulic, syringic, 4-coumaric, vanillic, and 4-OH benzoic acids were detected in wheat grain, bran, and aleurone fraction of bran (*21*, *22*). These phenolic acids may act as natural antioxidants and play important roles in the health benefits associated with consumption of whole-grain foods (*1-6*). Antioxidants may form chelating complexes with transition metals to reduce their availability as catalysts, and suppress the formation of the first few free radicals in the system to inhibit the initiation of the free radical-mediated oxidative chain reaction. Antioxidants may also directly react with and quench radicals in the system to terminate oxidative chain reactions. This

study examined radical scavenging properties of the five phenolic acids that are present in wheat grain and fractions, and their potential synergetic effects using spectrophotometric and ESR methods. ESR measures the presence of unpaired electron in the free radicals, and has been successfully used to study radical scavenging properties of antioxidants (*13*, *41*).

Interestingly, 4-OH benzoic acid exhibited no activity to react with and quench $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O_2^{\bullet-}$, or $HO^{\bullet}$ under the experimental conditions as measured by either ESR or spectrophotometric methods. This is in contrast to the previous observation that 4-OH benzoic acid at a concentration of 6.7 μM had greater $ABTS^{\bullet+}$ scavenging capacity than vanillic and syringic acids on a molarity basis (*32*). To confirm the $ABTS^{\bullet+}$ scavenging property of 4-OH benzoic acid, $ABTS^{\bullet+}$-antioxidant reactions were carried out with 4-OH benzoic acid at concentrations of 0.91, 2.3, 4.6, 9.1, and 91 μM. To further confirm the findings, the experiments were repeated using freshly purchased 4-OH benzoic acid from Sigma-Aldrich. No $ABTS^{\bullet+}$ scavenging activity was detected at any of these concentrations with two purchases of 4-OH benzoic acid. In addition, 4-OH benzoic acid was tested for its oxygen radical absorbing capacity (ORAC) since it showed strong ORAC in the previous study (*32*). The 4-OH benzoic acid exhibited strong ORAC, although it showed no $ABTS^{\bullet+}$ scavenging capacity. These data indicate that 4-OH benzoic acid is not capable to scavenge $ABTS^{\bullet+}$ at a greater activity than either vanillic or syringic acids. Also noted was that vanillic acid had greater $ABTS^{\bullet+}$ scavenging capacity than syringic acid as reported by Yeh and Yen (*32*), but syringic acid was a stronger $ABTS^{\bullet+}$ scavenger in the present study (Figure 5.5). Furthermore, results from the present study suggest that additional methoxyl group (OCH_3) in the ortho

position of the hydroxyl position on the phenyl ring enhances the $ABTS^{\bullet+}$ scavenging capacity of both 4-OH benzoic and 4-OH cinnamic acid derivatives. This is in contrast with Yeh and Yen's observation (*32*) that additional OCH_3 in the ortho position of the hydroxyl group on phenyl ring increased the $ABTS^{\bullet+}$ scavenging capacity of 4-OH cinnamic acid derivative, but decreased that of 4-OH benzoic acid derivatives. Theoretically, additional OCH_3 in the ortho position of hydroxyl group on phenyl ring increases the electron availability of the aromatic compound, and may act as a stronger electron donor, which may be associated with a greater $ABTS^{\bullet+}$ scavenging capacity. Additional evaluation from other independent laboratories is required to further confirm the $ABTS^{\bullet+}$ scavenging capacity of 4-OH benzoic acid.

In the present study, syringic acid exhibited stronger $O_2^{\bullet-}$ scavenging activity than vanillic acid, while ferulic acid had greater $O_2^{\bullet-}$ scavenging capacity than 4-coumaric acid (Figure 5.3), suggesting that additional OCH_3 in the ortho position of the hydroxyl group on phenyl ring also increase the $O_2^{\bullet-}$ scavenging capacity. The unshared pair of electrons of *o*-OCH_3 in *p*-orbital, as showed in the resonance structure III in Figure 5.10A and 5.10B, stabilizes the phenyl radical through electron delocalization and electron donation. It was also noted that ferulic acid had significantly greater capacity than vanillic acid in reacting with and quenching the $O_2^{\bullet-}$ in the system. This indicates that 4-OH cinnamic acid derivatives may have stronger $O_2^{\bullet-}$ scavenging activity than their corresponding 4-OH benzoic acid derivatives. This may be explained by the additional possible resonance structures of the resulting phenoxyl radicals of 4-OH cinnamic acids (Figure 5.10A and 5. 10B). This is supported by the observation of a previous study conducted by Natella and

others (*31*). Natella and others (*31*) evaluated the activities of benzoic acid and cinnamic acid derivatives in quenching peroxyl radical and suppressing LDL oxidation induced by either 2,2'-azobis(amidinopropane) dihydrochloride or Cu^{2+}, and concluded that the propenoic side chain could stabilize the phenoxyl radical by resonance and enhance the antioxidant activity of the phenyl ring (*31*).

ESR determination showed that only ferulic and vanillic acids had significant capacity to form chelating complex with Cu^{2+} among the tested phenolic acids under the experimental conditions (Figure 5.9). Both ferulic and vanillic acids contain OCH_3 in the ortho position of the hydroxyl group (Figure 5.1), suggesting that the presence of a hydroxyl and an OCH_3 in the ortho position might produce the formation of phenolic acid-Cu^{2+} chelating complex. This was different to the observation by Natella and others that only the phenolic acids with two hydroxyl groups in the ortho position showed Cu^{2+} chelating activity, measured by a spectrophotometric acid (*31*). This might be explained the greater sensitivity of ESR method and the weaker capacity of OCH_3 to interact with Cu^{2+} due to the steric effect. Interestingly, syringic acid with two ortho OCH_3 groups showed no activity to form chelating complex with Cu^{2+}, suggesting that addition of the second ortho OCH_3 on the phenyl ring dramatically eliminated chelating capacity of the phenolic acid. This might partially be explained by the change in the spatial arrangement of the hydroxyl group and the o-OCH_3 because of the steric exclusion, and possible interruption of electron delocalization between *p*- and π- orbitals.

In summary, five phenolic acids commonly present in wheat grain and fractions significantly differed in their properties to react with and quench $HO^{\bullet}$, $O_2^{\bullet-}$, $ABTS^{\bullet+}$, and

$DPPH^{\bullet}$. These phenolic acids also differed in their capacity to form chelating complexes with transition metals. Both substituents on the phenyl ring and the conjugated carbon skeleton may influence the antioxidant properties of phenolic acids. The presence of additional methoxyl group in the ortho position of the hydroxyl group enhance the radical scavenging capacity of phenolic acids against $O_2^{\bullet -}$, $ABTS^{\bullet +}$, and $DPPH^{\bullet}$, but not their $HO^{\bullet}$ scavenging activity. In addition, the presence of the first methoxyl group in the ortho position of the hydroxyl group on the phenyl ring may produce the capacity to form chelating capacity against transition metals, but introduction of the second methoxyl group to the ortho position of the hydroxyl group may eliminate the chelating capacity of the phenolic acid.

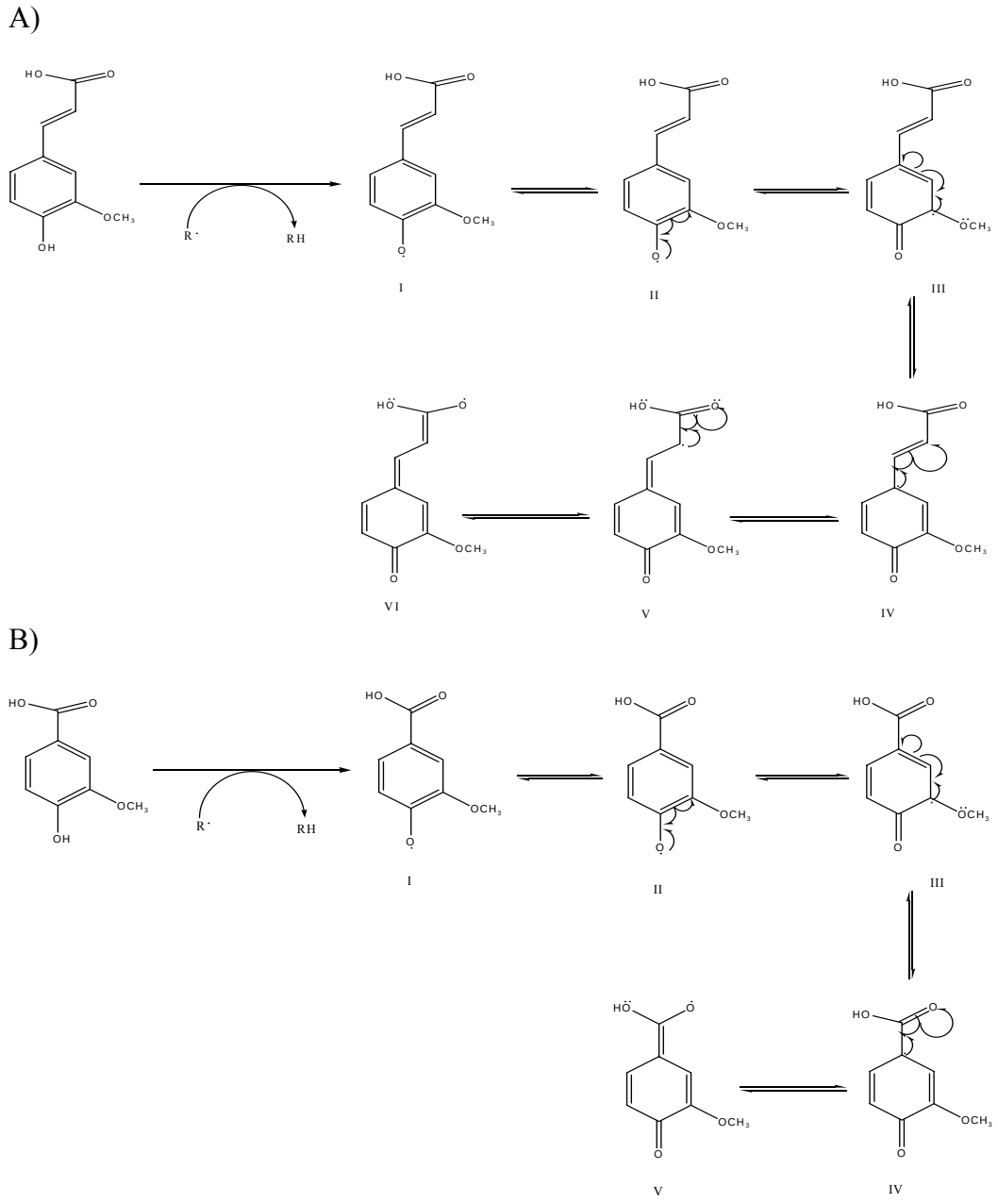


Figure 5.10. Phenoxyl radical formation and possible resonance structures for cinnamic and benzoic acid derivatives.

Phenoxyl radical is initially formed at the 4-OH group by abstraction of the hydroxyl H atom, regardless of the carbon skeleton of the molecule. **A)** represents the formation and resonance structures of 4-OH cinnamic acid derivatives including ferulic and 4-coumaric acids. For resonance structure III, the unshared pair electrons in the *P*-orbital of the 3-OCH_3 stabilizes the

unpaired electron on C3 of the phenyl ring. For resonance structure VI, the unshared pair electrons in the *P*-orbital of the hydroxyl group stabilizes the unpaired electron on O-atom of the carboxylic group. **B)** represents the formation and resonance structures of 4-OH benzoic acid derivatives including 4-OH benzoic, vanillic, and syringic acids. For resonance structure III, the unshared pair electrons in the *P*-orbital of the 3-OCH_3 stabilizes the unpaired electron on C3 of the phenyl ring. For resonance structure V, the unshared pair electrons in the *P*-orbital of the hydroxyl group stabilizes the unpaired electron on O-atom of the carboxylic group. Cinnamic acid derivatives may have six possible resonance structures for the phenoxyl radical, whereas benzoic acid derivatives have five possible resonance structures.

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