ABSTRACT

Title of Thesis: EFFECTS OF POST-HARVEST TREATMENT AND HEAT STRESS ON THE ANTIOXIDANT PROPERTIES OF WHEAT

Lan Su, Master of Science, 2006

Directed By: Professor Liangli (Lucy) Yu
Department of Nutrition and Food Science

This research examined and compared grain, bran, and micronized bran samples of Ankor and Trego wheat stored at 25, 60 and 100°C for their free radical scavenging properties, total phenolic content (TPC) and phenolic acid compositions. Bran and micronized bran samples stored at 100°C showed decreased TPC and free radical scavenging activities over 9 days of treatment, while there was no significant change observed for grain samples. Because of smaller particle size, micronized bran was more sensitive to heat stress when compared to bran at the two elevated temperatures. In addition, extractable phenolic acids were increased during storage for all kinds of samples on a per dry weight basis. These results demonstrate that whole grain is a preferred form of long-term storage for better antioxidant preservation, and reducing particle size may accelerate the loss of natural antioxidants in wheat bran during storage and heat treatment.

EFFECTS OF POST-HARVEST TREATMENT AND HEAT STRESS ON THE ANTIOXIDANT PROPERTIES OF WHEAT

By

Lan Su

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Advisory Committee:
Professor Liangli Yu, Chair
Professor Y. Martin Lo
Professor Jun-Jie Yin

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List of Abbreviations

AAPH	2,2'-azobis (2-amino-propane) dihydrochloride
ABTS	2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt
ACF	Aberrant crypt foci
ALS	Amyotrophic lateral sclerosis
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
CLA	Conjugated linoleic acid
$DPPH^•$	2,2-diphenyl-1-picrylhydrazyl radical
DTPA	Diethylenetriaminepentaacetic acid
EDTA	Disodium ethylenediaminetetracetate
ESR	Electron spin resonance
F-C reagent	Folin-Ciocalteu reagent
FL	Fluorescein
GE	Gallic acid equivalent
GSH	Glutathione
HAT	Hydrogen atom transfer
$HO^•$	Hydroxyl radical
HPLC	High performance liquid chromatography
HPX	Hypoxanthine
LDL	Low density lipoprotein
IQ	2-amino-3-methylimidazo[4,5- f]quinoline
NBT	Nitro blue tetrazolium
$O_2^{•-}$	Superoxide anion radical
ORAC	Oxygen radical absorbance capacity

RDA	Recommended daily allowance
RMCD	Randomly methylated beta-cyclodextrin
SOD	Superoxide dismutase
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic content
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
XOD	Xanthine oxidase

Introduction

Hard wheat is grown in the Great Plains of the United States and its flour is mainly used for making yeast breads and hard rolls since it has high protein level. Natural antioxidants are considered as possible value-adding factors for food products due to their potential health benefits and important role in maintenance of food quality and safety. Some wheat components showed substantial antioxidant activities and may be used as dietary antioxidants. Understanding the stability and availability of the wheat antioxidant during processing and storage is important for improved preservation and bioavailability of these antioxidants in hard wheat and wheat-based food ingredients for health benefits. This project evaluated and compared the stability and availability of wheat antioxidants in grain, bran, and micronized bran during storage under heat stress. The specific objectives are:

1. To evaluate the influence of heat stress on the antioxidant properties of grain, bran and micronized bran of Ankor and Trego wheat.
2. To investigate the potential effects of post-harvest treatment on the antioxidant availability of Ankor and Trego wheat.

The information obtained from this research will be used in determining the stability of wheat antioxidant after processing and storage in the food industry.

Chapter 1. Literature review

Potential roles of antioxidants in health

There are growing evidences suggesting that antioxidants may maintain health and prevent many chronic diseases, such as certain cancers, cardiovascular diseases and other aging-related diseases (*1-3*). Antioxidants may suppress the formation of free radicals, quench the existing radicals, and reduce the availability of oxygen in biological system to prevent the oxidative damage of DNA, proteins and lipids in human body (*3, 4*). Many studies have been performed to demonstrate their roles in health promotion and disease prevention.

Cancer prevention

Cancer is one of the leading causes of morbidity and mortality throughout the world. Biologically oxidative damage to DNA, lipids and proteins in the human body is normally considered to be one of the important factors in carcinogenesis (*3, 4*). Experimental data have established a wide range of biological effects of carotenoids, tocopherols, ascorbic acid, and selenium, many of which are thought to influence processes involved in carcinogenesis (*5, 6*). Reactive oxygen species appear to be involved in all steps of cancer development; as a result, dietary antioxidants may have potential benefits at different stages of carcinogenesis (*7, 8*). Epidemiological

studies have strongly proved a positive correlation between cancer-protective effects and high intakes of vegetables, fruits and whole grains (*8*). Further research should be investigated for those specific components in plants that are good for preventing certain cancers.

Cardiovascular disease prevention

Antioxidants may reduce the risk of coronary heart diseases (CHD) by inhibiting oxidative damage to arterial endothelial cells and circulating lipids (*9*). CHD are the leading cause of death in the United States as well as in most developed countries. It has been shown that oxidative damage to low-density lipoprotein (LDL) cholesterol greatly increases atherogenicity (*10*), and oxidized LDL has been shown to accelerate several steps in atherosclerosis including endothelial damage, uptake of LDL by foam cells, monocyte/macrophage recruitment, alteration in vascular tone, induction of growth factors, and production of antibodies (*11*). Dietary antioxidants maybe involved in the prevention of CHD through different mechanisms. For example, Vitamin E can inhibit oxidation of LDL cholesterol in plasma (*10*). Research in Europe also have reported inverse associations between plasma vitamin E levels standardized to plasma cholesterol levels and CVD mortality rates (*12, 13*). In contrast, β-carotene may prevent endothelial damage by decreasing LDL cholesterol uptake into cells (*14*). In addition, epidemiologic studies have found significant inverse relationships of per capita consumption of fresh fruits and vegetables rich in dietary antioxidants with CHD mortality in Great Britain (*15-17*).

Prevention of other diseases

Many studies showed that antioxidants may be effective for preventing or treating some aging-associated diseases, such as cataract, age related degeneration (AMD) and neurodegenerative diseases. Cataract and AMD are two common diseases of eyes recognized as the major cause of blindness in England, Canada and the United States (*18*). Evidence from human studies indicated that oxidation and antioxidant compromise induce degenerative ocular diseases. It is demonstrated that vitamin E, A, and C can prevent globular degeneration and reduce cataract and AMD by suppressing the oxidative damages to the ocular lens (*19-21*).

Examples of the neurodegenerative diseases include Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS). Oxidative stress may play a role either in triggering the defect or accelerating the onset of the disease after the defect happens. Although genetic defects are believed to be the main reason for these diseases, evidence indicates that oxidative stress including increased brain iron content, decline of superoxide dismutase (SOD) and glutathione (GSH), and oxidative damage to lipids, proteins, and DNA are important causes associated with neurodegenerative diseases (*22, 23*). In the last decade, several epidemiological and clinical studies evaluated the role of antioxidants, primarily vitamin E, in delaying the onset or treatment of these diseases (*24, 25*).

These results showed that dietary antioxidants may provide health benefits for neurodegenerative diseases.

Mechanisms of antioxidant defense

It has been known for a long time that a number of diseases are related to reactive oxygen species (ROS) (*26*). ROS, including superoxide anion ($O_2^{\bullet -}$), peroxide anion (O_2^{-}), hydroxyl radical ($OH^{\bullet}$) and singlet oxygen ($^1O_2$), are highly reactive molecules, formed from molecular oxygen (O_2) by gaining or losing electrons. ROS are produced spontaneously during biological processes, since they come from electron leakages from cell membranes and inadequately coupled reactions; the released electrons reduce molecular oxygen to superoxide anion or peroxide (*26*). There are several mechanisms about nutritional antioxidant defense and disease prevention, such as ROS scavenging, reduction of peroxides and repair of peroxidized biological membranes, and sequestration of iron to decrease ROS formation (*27-32*).

Natural antioxidants as food preservatives

In foods, free radicals cause autoxidation and development of rancidity. During the storage of food, transition metals, light or other factors may initiate the generation of free radicals leading to lipid peroxidation or degradation of proteins, vitamins and

pigments, cross-linked lipids and other macromolecules into non-nutritive polymers (*33*). As a result, lipid peroxidation leads to the formation of off-flavors, off-color and undesirable chemical compounds (*34, 35*). Antioxidants prevent foods from becoming rancid, browning, or developing black spots by suppressing the reaction that occurs when foods are exposed to oxygen in the presence of light, heat, and transition metals. Antioxidants also minimize the damage of essential amino acids and the loss of vitamins (*33*). Because of these benefits, antioxidants are used in food systems to improve food quality and stability.

Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are two common synthetic antioxidants and have been used as food preservatives for years, especially in foods rich in fats and oils. When the food additive amendment was enacted in 1958, BHA and BHT were listed as common preservatives considered generally recognized as safe (GRAS). GRAS regulations limit BHA and BHT to 0.02 percent or 200 parts per million (ppm) of the fat or oil content of the food product (Food additives amendment, 1958). While, later studies suggested that at very high levels (> 200 ppm), BHA induced tumors in the forestomach of rats, mice and hamsters, and liver tumors in fish (*36, 37*).

Due to the toxicity of synthetic antioxidant, dietary antioxidants are needed to replace BHA and BHT in food systems. For instance, sage, mace and black pepper extracts were used to inhibit the oxidation of frozen meat (*38*). Galangal and

rosemary extracts have been used to extend the shelf-life of minced beef (*39*). Tomato extracts have been found to be able to inhibit the singlet oxygen-catalyzed oxidation of α-linolenic acid and lipid peroxidation (*40*). In addition, tea polyphenolics have been used to control deterioration of food lipids (*41*). Due to the antioxidant beneficial roles in food systems and the toxicity of synthetic antioxidants, natural antioxidants are highly demanded.

Dietary antioxidants

Vitamin E

Vitamin E is a group of naturally occurring compounds including α-, β-, γ-, δ-tocopherols and tocotrienols (*42*). They are able to break the oxidative chain reaction in human body, prevent lipid peroxidation and protect cell membranes from free radical attack (*43, 44*). Vitamin E is one of the most effective lipid-soluble antioxidants, protecting unsaturated fatty acids in cell membranes that are important for keeping membrane function and structure (*44, 45*). Vitamin E can also improve the immune response and regulates platelet aggregation by inhibiting prostaglandin (thromboxane) formation (*46*). Additionally, it has a role in the regulation of protein kinase C (PKC) activation, mitochondrial function, nucleic acid and protein metabolism, and hormonal production (*46*). It has been reported that vitamin E protects vitamin A from destruction in the body and spares selenium (*46, 47*).

Generally, individuals with plasma vitamin E levels of less than 0.5 mg/dL are vitamin E-deficient. Daily dietary vitamin E intakes of 10-30 mg for healthy adults will maintain serum vitamin E concentrations in the normal range (*47*).

Vitamin C

Vitamin C including ascorbic acid and ascorbate, is a water-soluble vitamin found widely in plants. Its deficiency results in scurvy, a disease with an insidious onset, but fatal results. Ascorbate is an electron donor, and this property accounts for antioxidative functions (*48*). As an antioxidant or reducing agent, it sequentially donates two electrons from the C2-C3 double bond, forming the intermediate ascorbate free radical. This free radical is unstable and can be reversibly reduced to ascorbate (*48*). Vitamin C can scavenge reactive oxygen species, thereby effectively protecting other substrates from oxidative damage. The reactive oxygen species scavenged by vitamin C include superoxide and aqueous peroxyl radicals, singlet oxygen, and ozone (*4*). In addition to scavenging these reactive species, vitamin C can interact with other small-molecule antioxidants, such as tocopherol, glutathione and β-carotene, and inhibit their oxidation (*4*).

Carotenoids

Carotenoids are a large group of compounds, including lycopene, β-carotene, α-carotene, β-cryptoxanthin, zeaxanthin, lutein, canthaxanthin and astaxanthin with

various structural features and biological actions. They may compete or act synergistically with each other, or with other protective components in foods and biological systems. Dietary carotenoids have been applied in biological processes that may have been related to human health and chronic disease. Those biological actions of carotenoids include antioxidant activity, intercellular communication, cell differentiation, immunoenhancement, and inhibition of mutagenesis and transformation (*49*). Many epidemiological studies have supported an inverse association between dietary intake or blood levels of carotenoids and risk of several chronic diseases, such as cancer, heart disease, cataracts, diabetes and macular degeneration and Alzheimer's disease (*50*). The antioxidant capacity of carotenoids is mainly due to conjugated double bonds, a structural feature that allows carotenoids to quench or inactivate some highly reactive molecules. Therefore, carotenoids are good quenchers of singlet oxygen species and can directly scavenge free radicals (*51, 52*), which may be one of the reasons for their role in preventing potential diseases. The system of conjugated double bonds can also be associated with pro-oxidant activity under certain conditions (*53*).

A growing number of epidemiological evidence has suggested a role for fruits and vegetables, rich in β-carotene, in the prevention of lung carcinogenesis (*3*). β-carotene is both a chain-breaking antioxidant and a quencher of singlet oxygen. It accumulates in lung and could partially reverse the oxidative damage in lung tissues (*54*). Recently, epidemiological studies also have shown that high intakes of

tomatoes and tomato products as well as high blood levels of lycopene are associated with a decreased risk of prostate cancer (*54-56*). Strong scientific evidence supports the protective role of lutein and zeaxanthin in age-related eye diseases (*57, 58*) because they accumulate in the pigment of the macular region of the normal retina (*59, 60*). In addition, research indicated that carotenoids have effects on skin cancer, cardiovascular disease and immune response (*61*).

Polyphenols and flavonoids

Polyphenols are a largest category of phytochemicals, an integral part of the human diet, and most widely distributed in the plant kingdom. Dietary phenolics include phenolic acids, phenolic polymers (commonly known as tannins), and flavonoids (*61-63*). Flavonoids compose the largest and most-studied group of plant phenols. Over 4000 different flavonoids have now been identified, and their molecular structures consist of an aromatic ring A, condensed to heterocyclic ring C, and attached to a second aromatic ring B (*64*). They may contain several phenolic hydroxyl groups attached to the aromatic rings, which confer their potent antioxidant activity. Polyphenols and most flavonoids are powerful antioxidants, which act as reducing agents, proton-donators and singlet oxygen quenchers, and may be used for medicinal and dietary supplemental purposes (*64, 65*). Some polyphenols also act as metal ion chelating agents, thereby reducing its availability for generating free radicals (*65*). Structure-activity studies revealed that the antioxidant efficiency of flavonoids is directly related to their degree of hydroxylation (*64*). Flavonoids are

proved to be effective scavengers of hydroxyl and peroxyl radicals, and superoxide anion (*66-68*).

Wheat as a good dietary source of antioxidants

Wheat - a mainly daily dietary component

Wheat or wheat-based food has been a staple food for humans throughout recorded history. Wheat is a major diet component because of its adaptability, ease of storage and converting grain into flour for making edible, palatable, and satisfying foods. Most of the wheat varieties cultivated today can be grouped into two general categories: hard and soft wheat. Hard wheat is used for making bread which accounts for around 95% of the current world production; the other remaining 5% is soft wheat used for producing biscuits or cakes, etc (*69*). Hard wheat grain usually has a hard kernel and high level gluten and protein. Soft wheat grain, on the other hand, has a lower protein level. A third group between hard and soft wheat also exists. It is called semi-hard wheat which has some above properties and used in unleavened breads such as Asian steamed bread and certain noodles (*69*). Wheat could be further sorted by color and growth season. Color of wheat (e.g., red or white) means the color of the aleurone or outer layer of the wheat kernel. Different wheat color may reflect specific nutritional value of the grain (*69*). In addition,

wheat could be also classified into spring or winter wheat according to growth season (*69*).

Wheat is the most important source of carbohydrate in human diet. It is estimated that nearly two-thirds of the wheat produced in the world is used for food; the remaining one-third is used for feeding, seed and non-food applications (*70*). Wheat could provide a major source of energy, protein and dietary fiber for human nutrition. Wheat based food products contribute about 20% of the energy for human diet (*71*). This is an underestimate of total contribution, as wheat used in livestock also supplied indirectly. Wheat is also a good source of protein which contributes over 25% of the protein of the human diet (*72*). Wheat grain itself has low level of fat, and the fat is usually unsaturated fatty acids which could lower blood cholesterol levels and prevent heart disease (*72*). Whole wheat grain and its bran fraction are good sources of dietary fiber, particularly water insoluble fiber, which is good for promoting fecal bulk and reducing the risk of colorectal cancer (*72*). In contrast, wheat flour has certain level of soluble fiber and could help normalize blood cholesterol and sugar level (*72*).

Wheat - a good source of dietary antioxidants

There is positive relationship between the consumption of whole-grain products and reducing the incidence of chronic diseases (*73-76*). These beneficial effects have been attributed to the unique phytochemicals of the grains. In 1972, Fulcher first

found that wheat grain contained antioxidants (*77*). Since then, a number of studies have detected antioxidant properties in wheat and wheat-based food products. It is widely accepted that phenolic acids including ferulic, vanillic, and *p*-coumaric acids are the major antioxidants in wheat and significantly contribute to the overall antioxidant properties of wheat grain (*78*). In addition to the phenolic acids, carotenoids were also found in wheat including lutein, zeaxanthin, and β-crypoxanthin which varied significantly among different varieties (*79-81*). Tocopherols are another group of well-known antioxidants in wheat grain and bran which are good for health (*2, 79, 82*). Zhou and others (*81*) detected that the concentrations of α-, δ-, and γ- tocopherols were 1.28-21.29, 0.23-7.0, and 0.92-6.90 μg/g in wheat bran, respectively.

Wheat antioxidants could directly react with or quench free radicals, suppress lipid peroxidation in fish oil and liposome, and inhibit several chronic diseases (*78, 83-85*). Winter wheat grain extracts inhibited radical-induced liposome lipid peroxidation and showed radical cation scavenging activity (*86*). Previous study showed that Durum wheat bran extract (Triticum durum) suppressed oil oxidation (*78*). Wheat bran extract had inhibitory effects on human LDL oxidation and scavenged free radicals (*87*). In animal studies, some research indicated that higher intake of whole wheat grains could lower the risk of colon cancer (*74, 88*). Wheat bran was found to reduce the incidence of colon tumors in rats given azoyxmehtane (AOM) (*89*). Dietary supplementation with wheat bran protected against the

formation of colonic aberrant crypt foci (ACF) induced by 2-amino-3-mehtylimidaze [4, 5-f] quinoline (IQ) in the rat (*90, 91*). These finding indicated that wheat could be a potential source of natural antioxidants for food preservation and health promotion.

Antioxidants - value-adding factors for wheat production and trade

Wheat production and trade are in the leading agricultural position, followed by rice, maize and potatoes (*69*). Among wheat produced in the USA, only four-fifth is consumed, the others is exported to developing countries. Four largest exporting countries include the United States, Canada, France and Australia (*92*). During the 2004-05 marketing year, a decrease of exporting wheat to other countries were observed in the American wheat market (*93*). Wheat producers have to find other marketing opportunities for the wheat in order to increase their marketing values and enhance the agricultural economy because of a current depression in the U.S. and exported wheat markets, and the growing competition from other crops (*94*).

The antioxidant is a group of health beneficial components in wheat grain that consumers often neglect. People paid more attention on the fruit and vegetables than wheat for their antioxidant benefits although wheat products are in big needed in the food guide pyramid. In the United States, 90% consumers are not taking the recommended 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 (*95*). Thus, if we could demonstrate wheat is a high-antioxidant food ingredient, wheat consumption would increase because of its health benefits. This benefit may stimulate agricultural economy and increase farmer income by promoting value added production and utilization of wheat for health promotion and food applications.

Effect of thermal treatment on the antioxidant properties of foods

Food producers become increasingly interested in developing new products with certain health-promoting compounds. For this purpose, it is important to know the stability of beneficial food components during processing. Several studies were carried out to study thermal and storage stability of antioxidant properties in food. It is well known that naturally occurring antioxidants could be significantly lost during processing and storage (*64*). In particular, thermal processing is believed to be the main cause of the depletion for antioxidants in natural foods (*96*). Previous study found that the content of ascorbic acid, total anthocyanins, and the hydrophillic antioxidant capacity in strawberries decreased during heat treatment (*97*). Polydera and others (*98*) found that the total antioxidant activity of orange juice decreased during storage because of loss of ascorbic acid. Another study taken on barley showed that the antioxidant efficiency of the roasted samples was weaker than that of unroasted samples because of the reduction of antioxidant components (catechin, tocopherol, and lutein) under the increased roasting temperature (*99, 100*).

However, some studies indicated that thermal processing may not influence antioxidant property of samples or even develop new antioxidants and increase total antioxidant content (*101*). Sluis (*102, 103*) observed that long-term storage under different conditions didn't alter flavonoid concentration or antioxidant activity in apples. Dewanto (*104*) found that thermal processing elevated total antioxidant activity and bioaccessible lycopene content in tomatoes and produced no significant changes in the total phenolics and total flavonoids content, although loss of vitamin C was observed. The lycopene content in tomato increased from 2.01 to 3.11, 5.45 and 5.32 trans-lycopene/g dry tomato after 2, 15, 30 min of heating at 88°C, respectively. The antioxidant activity of tomatoes enhanced from 4.13 µmol of vitamin C equiv/g of tomato to 5.29, 5.53 and 6.70 µmol vitamin C equiv/g of tomato, respectively (*104*). A kinetic study showed that the water soluble antioxidant capacities of both orange and carrot juice increased in the first 60 min and 90 min of thermal treatment at 75-120°C, separately (*105*). Antioxidant properties of tomato juice decreased after short-term heat treatments and a recovery of these properties by prolonging heating time (*106*). These observations were explained by some previous studies suggesting possible thermal degradation of naturally occurring antioxidant properties and the potential formation of Maillard reaction products (MRP) with antioxidant properties may attribute the overall antioxidant activity (*106-108*). These studies indicated that some chemical reactions may happen and influence the antioxidant availability and stability of food.

Effect of post-harvest treatment on the antioxidant stability of wheat fractions

Human consumption of wheat is mainly from products milled from the whole grain. The starchy endosperm has 72% of the protein in the grain and provides 99% of the flour used to make white bread. The bran is made up of the pericarp, testa and aleurone. It represents 20% of the protein and is a good source of dietary fibre, minerals and other phytochemicals (*72*). Ferulic acid is one of the major phenolic acids in wheat whole grain and was found in high concentration in the aleurone cell walls, but not in significant quantities in the starchy endosperm of the mature grains (*109*). Pussayanawin and Wetzel (*110*) observed that phenolic compounds are concentrated in the aleurone and bran portions of cereal kernels. Onyenebo and Hettiarachchy (*78*) also found that extract from durum wheat bran showed stronger antioxidant activity than extracts from other milling fractions. In addition, bran fractions of wheat had 15-18 fold more total phenolic content, 4-fold more lutein, 12-fold more zeaxanthin, and 2-fold more β-cryptoxanthin than that of respective grain fractions (*111*). These results indicated that wheat bran is a better antioxidant resource than wheat grain.

The chemical composition and bioavailability of nutrients varies between varieties of wheat and may be affected by forms of post-harvest treatment. Modern milling processes involve a complex assay of grinding, separating and mixing procedures to

get a multitude of flour grades with different particle size. Zhou et al. (*112*) found that micronized aleurone, aleurone, and bran may significantly differ in their antioxidant properties, TPC, and phenolic acid composition. Among them, micronized aleurone had the highest antioxidant properties, indicating the possibility of post-harvest treatment on antioxidant stability and availability of wheat or wheat-based food.

Antioxidant property assays

Antioxidants are chemically diverse, which containing vitamin C and E, carotenoids, polyphenols and flavonoids present in plant-based food. Wheat antioxidants mainly include phenolic acids, tocopherols and carotenoids. Due to the complexity of the composition of foods and the possible synergistic interactions among the antioxidant compounds in a food mixture, it is hard to separate each antioxidant compound and study them individually (*113*). Therefore, it is important to use a convenient method that can measure the antioxidant capacity directly from food extracts.

There are two kinds of mechanisms of free radical scavenging capacity assay: the first one based on hydrogen atom transfer (HAT) reaction and the second one based on electron transfer (ET) reaction (*113*). Most of the HAT-based assays apply a competitive reaction scheme, in which antioxidant and substrate compete for synthetic generated radicals. These methods generally involve a synthetic free

radical generator, an oxidizable molecular probe, and an antioxidant. The HAT-based assays include inhibition of induced lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP) and crocin bleaching assays (*113*). ET-based assays involve one redox reaction with the oxidant as an indicator of the reaction endpoint and determine the capacity of an antioxidant in reducing an oxidant, which changes color when reduced. The degree of color change is proportional to the sample's antioxidant concentration. ET-based assays include the total phenols assay, trolox equivalence antioxidant capacity (TEAC), ferric ion chelating activity (FRAP), and 2,2-Diphenyl-1-picrylhydrazyl $DPPH^{\bullet}$ radical scavenging capacity assay (DPPH) (*113*). In addition, there are other assays intended to measure a sample's scavenging capacity of biologically relevant oxidants such as superoxide anion and hydroxyl radicals (*113*).

Oxygen radical absorbance capacity assay (ORAC)

The oxygen radical absorbance capacity assay (ORAC) determines the peroxyl radical scavenging capacity of samples using a competitive assay. In the reaction, antioxidants compete with the molecular probe fluorescein (FL) for peroxyl radical and are degraded by peroxyl radicals generated by the thermal decomposition of an azo compound (AAPH) (*113*). This antioxidant "scavenging" of peroxyl radicals spares the FL from degradation which decreases its fluorescence. This "scavenging" capacity is calculated using the area under the curve (AUC). Results are expressed

as trolox equivalents (a water soluble vitamin E derivative) with the units of μmoles trolox per gram material for natural botanical extracts (*113*).

Cutler and Cao (*114-116*) improved ORAC assay providing a direct measure of the hydrophilic and lipophilic chain-breaking antioxidant capacity versus peroxyl radicals. ORAC assay has the following components: (a) an azo radical initiator, normally AAPH; (b) a molecular probe (UV or fluorescence) for monitoring reaction progress; (c) antioxidant; and (d) reaction kinetic parameters collected for antioxidant capacity quantification. Huang described the detailed procedures of ORAC running on a 96-well plate fluorescence reader for highthrouput analysis (*117*). In summary, samples, controls, and standard (trolox of four or five different concentrations for construction of a standard curve) are mixed with fluorescein solution and incubated at constant temperature (37°C) before AAPH solution is added to initiate the reaction. The fluorescence intensity is measured every minute for 40 min at pH 7.4 and 37°C. The fluorescien is consumed and FL intensity decreases as the reaction progresses. If the antioxidant is present, the FL degradation is delayed. The ORAC assay has been broadly applied in academics and the food and supplement industry as a method of choice to quantify antioxidant capacity.

Trolox equivalent antioxidant capacity assay (TEAC)

ABTS is the acronym for 2,2' azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt. Miller and Rice-Evans first reported TEAC in 1993 and improved it later (*118*). In the improved version, $ABTS^{\bullet+}$, the oxidant, was generated by manganese oxidation of 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{2-}$). The activated radical is a blue colored radical with max absorption at 734 nm. The ability of antioxidants to decolorize the $ABTS^{\bullet+}$ radical (i.e. scavenge the radical) is then determined. Trolox of four or five different concentrations are used for construction of a standard curve. One milliliter of the $ABTS^{\bullet+}$ solution was mixed with 80 μL of sample extracts. The absorbance was read at 1.5 min after 30 second vortex mixing. Due to its simplicity, the TEAC assay has been used in many laboratories for studying antioxidant capacity (*119*).

2,2-Diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical scavenging capacity assay

$DPPH^{\bullet}$ is one of a few stable and commercially available organic nitrogen centered radicals and has a UV-vis absorption maximum at 517 nm. The solution color fades as the reduction progress and the reaction is easily monitored by a spectrophotometer (*113*). The $DPPH^{\bullet}$ scavenging capacity assay is technically simple, but some disadvantages limit its applications. $DPPH^{\bullet}$ is a long-lived nitrogen radical, which has no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Therefore, many antioxidants that react

quickly with peroxyl radical may react slowly or may even be inert to $DPPH^{\bullet}$ (*120*). Foti (*121*) found that the rate-determining step for the DPPH reaction consists of a fast electron transfer process from the phenoxide anions to $DPPH^{\bullet}$ and behaved like ET reaction.

Superoxide anion ($O_2^{\bullet -}$) radical scavenging activity by ESR

The superoxide anion radicals are produced by a hypoxanthine (HPX)-xanthine oxidase (XOD) system. The addition of XOD initiates the reaction, and DMPO was used as the trapping agent. The reaction mixture consists of HPX, 5,5-dimethyl-1-pyrroline N-oxide (DMPO), diethylenetriaminepentaacetic acid (DTPA), superoxide dismutase (SOD)/sample and XOD. All reagents are dissolved in 5 mM potassium phosphate buffer (pH 7.4). In this reaction, the DMPO-OOH spin adducts are detected by ESR spectra. The ESR signal intensity of the $O_2^{\bullet -}$ is proportional to its concentration in the reaction system. The radical $O_2^{\bullet -}$ scavenging activities were determined by comparing with the intensity of the $O_2^{\bullet -}$ signal in extracting solvent (*122, 123*).

Total phenolic content (TPC) assay by Folin-Ciocalteu reagent

Singleton first extended total phenols assay by Folin-Ciocalteu reagent (FCR) to the analysis of total phenols in wine (*127*). The FCR react with reducing agents, but this does not reflect in the name ‘total phenolic content’. Lots of studies found good

linear correlations between the 'total phenolic profiles' and 'the antioxidant activity'. The FCR is prepared by the following procedures. First, the mixture of sodium tungstare, sodium molybdate, concentrated hydrochloric acid, 85% phosphoric acid and water is boiled for 10 hours. Then, lithium sulfate is added to the mixture to give an intense yellow solution- the FC reagent. Finally, bromine is added into the mixture to restore the desired yellow color as oxidant. The exact chemical nature of the FCR is not known, but it may contain heteropolyphosphotunstates-molybdates (*123*). The reduction reaction leads to blue species at last. Evidently, the FCR is not specific to phenolic compounds and it can be reduced by many nonphenolic compounds (e.g., vitamin C). Only under basic solutions, FCR reacts with phenolic compounds (adjusted by a sodium carbonate solution to pH ~10). Although the nature of FCR is undefined, the total phenols assay by FCR is convenient, reproducible, and has become a routine assay in studying phenolic antioxidants (*113*).

Specific objectives and significance of this research

Hard wheat and wheat based food are important dietary sources of antioxidants and have many potential health benefits (*1-4*). However, antioxidant may be lost during post-harvest treatment and thermal stress (*96*). Understanding of the antioxidant stability and availability of the wheat and wheat-based food during storage and under heat stress is important for better food preservation and improved

bioavailability of these antioxidants in hard wheat and wheat-based food ingredients for health benefits. Our preliminary study showed decreased $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging activities of grain, bran and micronized bran of Ankor wheat treated at 25, 60, 100°C for five days. Therefore, the objective of this research was to further evaluate and compare the stability and availability of wheat antioxidants in grain, bran, and micronized bran of Ankor and Trego wheat during storage and under heat stress.

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Chapter 2. Effects of post-harvest treatment and heat stress on the antioxidant properties of wheat

Abstract

Grain, bran, and micronized bran samples of Ankor and Trego wheat stored at 25, 60 and 100°C were examined and compared for their free radical scavenging properties against peroxyl radical (ORAC), $ABTS^{\bullet+}$, 2,2-diphenyl-1-picryhydrazyl ($DPPH^{\bullet}$), and $O_2^{\bullet-}$. In addition, total phenolic content (TPC) and phenolic acid compositions were also determined. Bran and micronized bran samples stored at 100°C showed decreased TPC and scavenging activities against peroxyl, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals over 9 days of treatment, while there was no significant change observed for grain samples. Because of smaller particle size, micronized bran was more sensitive to heat stress when compared to bran at the two elevated temperatures. In addition, extractable phenolic acids including vanillic, syringic, *p*-coumaric and ferulic acids were increased during storage for all grain, bran and micronized bran samples on a per dry weight basis. These results demonstrate that whole grain is a preferred form of long-term storage for better antioxidant preservation, and reducing particle size may accelerate the loss of natural antioxidants in wheat bran during storage and heat treatment.

Introduction

Wheat is an important food component in human diet and a major agricultural commodity across the world. Epidemiological studies have related the consumption of whole wheat and whole-wheat products to the reduction of chronic diseases, such as heart disease, cancer and age-related degenerative diseases (*1-5*). Antioxidants in wheat may contribute to these health benefits by quenching free radicals, inhibiting the formation of free radicals, and reducing the availability of oxygen in biological systems to prevent the oxidative damage to proteins, lipids and DNA (*6-8*). Previous studies have shown that the extracts of wheat and wheat-based products exhibited radical scavenging capacities, suppressed lipid peroxidation in oils and human low-density lipoprotein (LDL), and showed chelating activity against Fe^{2+} (*9-16*). In addition, unique phytochemicals in wheat grain and bran have also been determined. Experimental data demonstrated that ferulic acid was the predominant phenolic acid in all of the tested bran samples and accounted for approximately 46-67% of total phenolic acids on a per weight basis (*15*). Other relevant phenolic acids included syringic, *p*-hydroxybenzoic, vanillic and *p*-coumaric acids (*15*). In addition to phenolic acids, tocopherols and carotenoids were also found in grain and bran samples of different wheat varieties(*15, 17-19*). However, wheat is normally consumed after processed to wheat-based food following post-harvest processing or thermal treatment which may alter the stability and availability of wheat antioxidants.

It is well accepted that naturally occurring antioxidants could be lost as a result of post-harvest treatment and/or storage. In particular, heat treatment is commonly believed as the main cause of dietary antioxidant depletion (*20, 21*). Klopotek and co-workers (*22*) found that the content of total anthocyanins and ascorbic acid in strawberry decreased during heat treatment. Orange juice was also shown to have a corresponding reduction in ascorbic acid and total antioxidant activity during storage (*23*). However, other researches showed that heat treatment may not affect antioxidant properties or even developed new antioxidants and increased antioxidant activity (*24*). For example, the flavonoid content or antioxidant capacities of apples did not significant change over long-term storage under different conditions (*25*). Dewanto and co-workers (*26*) found that thermal processing increased total antioxidant activity and bioaccessible lycopene content in tomatoes and showed no significant changes in the total phenolic and total flavonoid contents, although loss of vitamin C was found. These results indicated that the thermal degradation of naturally occurring antioxidants and the formation of Maillard reaction products (MRP) with antioxidant properties may attribute to the overall antioxidant activities (*27-29*).

To date, there has been little research investigating the effect of post-harvest treatment and heat stress on the antioxidant properties of hard winter wheat varieties. In the present study, 100% ethanol extracts of different wheat fractions of Ankor and Trego wheat grain stored at three temperatures were evaluated for their

free radical scavenging activities, total phenolic content and phenolic acid compositions.

Materials and Methods

Chemicals and reagents. 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picryhydrazyl radical ($DPPH^{\bullet}$), fluorescein (FL), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), diethylenetriaminepentaacetic acid (DTPA), hypoxanthine (HPX), and xanthine oxidase (XOD) 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). 5-*tert*-Butoxycarbonyl 5-methyl-1-pyrroline N-oxide (BMPO) was a gift from Professor B. Kalyanaraman of the Biophysics Research Institute and Free Radical Research Center at the Medical College of Wisconsin (Milwaukee, WI). All other chemicals and regents were of the highest commercial grade and used without further purification.

Sample preparation. Grain, bran and micronized bran samples of Ankor and Trego, two hard winter wheat varieties grown in Colorado and the west central Great Plains, were examined in this study. Samples were provided by Dr. Scott Haley of the Department of Soil and Crop Science, Colorado State University, Fort Collins, CO. Micronized bran was prepared by grinding samples using a Bel Art micromill (Pequannock, NJ) to pass through a 40 mesh screen.

Heat treatment. To examine and compare the stabilities of wheat antioxidants in those samples under heat stress, 2-3 gram of individual samples (grain, bran and micronized bran) of Ankor and Trego wheat was placed in an aluminum dish and stored at 25, 60 and 100°C. For each thermal treatment, samples were taken at the 0, 1, 2, 3, 5 and 9 days of storage. After removing the dishes from the thermal storage, the samples were immediately transferred to a desiccator and then kept in sealed bag until the extraction was performed. Dishes and samples were accurately weighed before and after each temperature treatment, and the moisture content was calculated for all original grain, bran and micronized bran. Samples stored at the ambient temperature (25°C) for 0 day were used as references. Treatments were conducted in triplicate.

Extraction preparation. Grain and bran samples of Ankor and Trego were ground to fine powder. One gram of each ground sample was extracted with 10 mL 100% ethanol for 15 h under nitrogen at ambient temperature. All extracts were stored in dark under nitrogen at ambient temperature until further analysis.

Oxygen radical absorbing capacity (ORAC). ORAC assay was conducted using fluorescein (FL) as the fluorescent probe and a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland) according to a laboratory protocol (*30*) with slight modifications. Standards were prepared in 100% ethanol while all other reagents were prepared in 75 mM sodium phosphate buffer (pH 7.4). Initial reaction mixture contained 225 μL of 8.16×10^{-8} M FL, 30 μL sample, standard, or 100% ethanol for blank, and 25 μL of 0.36 M AAPH. FL and samples were mixed in 96-well plate

and pre-heated in plate reader for 20 min at 37ºC after which the AAPH solution was added. The fluorescence of the assay mixture was recorded every minute for 45 min at 37ºC. Excitation and emission wavelengths were 485 nm and 535 nm, respectively. Trolox equivalents (TE) were calculated for samples based on the same AUC calculations used for the ORAC assay (*31*) with results expressed as μmoles of TE per gram wheat on a per dry weight basis. One hundred percent ethanol was used to prepare TE standard and blank.

Radical cation ABTS$^{\bullet+}$ scavenging activity. Radical scavenging capacities of wheat grain, bran and micronized bran extracts were evaluated against ABTS$^{\bullet+}$ generated by the chemical method based on a previous published protocol (*15, 32*). In brief, ABTS$^{\bullet+}$ was generated by oxidizing 5 mM aqueous solution of ABTS with manganese dioxide at ambient temperature for 30 min. The testing samples were prepared by diluting 50 μL of wheat extracts with 450 μL of 7% RMCD. The reaction mixture consisted of 1.0 mL of ABTS$^{\bullet+}$ and 80 uL of antioxidant testing samples. The initial absorbance of ABTS$^{\bullet+}$ at 734 nm is 0.7. The absorbance of reaction was measured at 1.5 min of the reaction, and trolox was used to prepare standard curve.

Radical DPPH$^{\bullet}$ scavenging activity. The free radical DPPH$^{\bullet}$ scavenging activity of wheat extracts was measured according to the previously reported method using the commercially stable 2,2-diphenyl-1-picryhydrazyl radical (DPPH$^{\bullet}$) (*12*). The final concentration of DPPH$^{\bullet}$ was 100 μM, and the final reaction volume was 2.0 mL. The absorbance of wheat extract-radical reaction at 517 nm was determined

against a blank of 100% ethanol at 5 min. The absorbance of 100% ethanol - DPPH$^{\bullet}$ radical reaction was used as control.

Superoxide anion radical ($O_2^{\bullet -}$) scavenging activity. Superoxide anion radical ($O_2^{\bullet -}$) scavenging activity was measured by an electron spin resonance (ESR) method using the $O_2^{\bullet -}$ produced from the xanthine/xanthine oxidase system. The reaction mixture was 2 mM xanthine, 100 mM DMPO, 0.2 mM DTPA, 0.08 unit/mL of xanthine oxidase solution (XOD), and 20 mg/mL sample extracts. The total volume was 100 μL. XOD solution was used to initiate the antioxidant-radical reaction, and DMPO was used as the trapping agent (*33*). The ESR spectra were recorded from 2-5 min of reaction with 10 mW incident microwave power and 100 kHz field modulation of 1 G at room 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 U.S. Food and Drug Administration (College Park, MD). The ESR signal strength of the $O_2^{\bullet -}$ is proportional to its concentration in the testing system. The radical $O_2^{\bullet -}$ scavenging activities were determined by comparing with the strength of the $O_2^{\bullet -}$ in extracting solvent.

Total phenolic content (TPC). The total phenolic content of wheat extracts were determined using Folin-Ciocalteu reagent (*12*). Known amounts of ethanol wheat extracts were obtained under vacuum and the solid residue was quantitatively redissolved in DMSO. The resulting DMSO solution was used in TPC assay. The Folin-Ciocalteu reagent was prepared according to a previous protocol (*12*). The reaction mixture contained 50 μL of sample extract, 250 μL of Folin-Ciocalteu

reagent and 750 uL of 20% sodium carbonate. After 2 h of reaction at room temperature, the absorbance at 765 nm was determined and used to calculate TPC taking gallic acid as a standard.

Phenolic acid composition. The wheat extracts were analyzed for their phenolic acid profile. After removing 100% ethanol with nitrogen, the wheat antioxidants were hydrolyzed with 2N NaOH for 4 h at 50°C, acidified using 6N HCl, and extracted with ethyl ether-ethyl acetate (1:1, v/v) according to the previous procedure (*15, 34*). The ethyl ether-ethyl acetate was removed at 35° using a nitrogen evaporator, and the solid residue was redissolved in methanol-tetrohydrofuran (1:1, v/v), filtered through a 0.45 µm membrane filter, and kept refrigerated at 4°C until high-performance liquid chromatography (HPLC) analysis for phenlic acids. The phenolic acid composition in the solution was determined by reverse-phase HPLC with a Phenomenex C_{18} column (250 mm x 4.6 mm). The phenolic acids were detected at 280 nm and 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 (*15*). Identification and determination of phenolic acids composition in each solution was accomplished by comparing the retention time of peaks and the total area of each peak in wheat extracts to that of the phenolic acid standard.

Statistical analysis. Data were reported as mean ± SD for triplicate determinations. Analysis of variance ANOVA and least significant difference tests

were applied to identify differences among means, while a Pearson Correlation test was conducted to analyze the correlation among means (SPSS for Windows, Version. 10.0.5., 1999, SPSS Inc., Chicago, IL). Statistical significance was declared at $P < 0.05$.

Results

Oxygen radical absorbing capacity (ORAC). ORAC assay measures the ability of wheat extracts for scavenging peroxyl radical. The ORAC values were determined for 100% ethanol extracts of Ankor and Trego wheat samples, and expressed as micromoles of trolox equivalent (TE) per gram of wheat. Comparing grain and bran of Ankor and Trego, all the extracts showed significant ORAC values (Table 1). The highest ORAC value was 39.62 μmoles of TE/g wheat for Trego bran (Table 1). The lowest ORAC value was seen in Trego grain. Wheat bran had greater ORAC value than grain, indicating that antioxidants of wheat are mainly distributed in the bran.

Table 1. Antioxidant properties of Ankor and Trego wheat at Day 0*.

		ORAC	ABTS	DPPH	TPC
		(TE µmoles/g wheat)	(TE µmoles/g wheat)	% remaining	(GE mg/g wheat)
Ankor	Grain	3.74 ± 0.92a	5.74 ± 0.08a	62.30 ± 0.47c	0.36 ± 0.03a
	Bran	12.73 ± 4.75b	14.39 ± 0.75c	35.62 ± 2.21a	0.80 ± 0.05c
Trego	Grain	3.07 ± 0.40a	4.39 ± 0.17a	72.58 ± 2.33d	0.36 ± 0.00a
	Bran	39.62 ± 3.02c	8.32 ± 0.17b	54.05 ± 0.44b	0.54 ± 0.03b

* ORAC, Oxygen radical absorbing capacity; ABTS, $ABTS^{\bullet +}$ radical scavenging properties; DPPH, % $DPPH^{\bullet}$ quenched was determined at 5 min of the antioxidant-$DPPH^{\bullet}$ radical reaction; TPC, total phenolic content; TE, Trolox equivalent; GE, Gallic acid equivalent.

The grain, bran, and micronized bran of Ankor and Trego wheat stored at 25, 60, and 100°C for up to nine days were analyzed for their antioxidant activities (Figure 1). Figure 1 shows the ORAC values of wheat samples subjected to different heat treatments for various days. There were no significant changes in scavenging activity against peroxyl radical in grains stored at all three temperatures for both Ankor and Trego wheat. For bran and micronized bran heated at 100°C, the samples exhibited reduction in their antioxidant activities. The ORAC value of micronized bran decreased more than bran at 100°C indicating that micronized bran was more

sensitive to heat treatment than bran (Table 2). Interestingly, bran samples stored at 60°C had higher ORAC scavenging activities than those kept at 25 and 100°C. Although Ankor and Trego showed different ORAC values without heat treatment, they had similar antioxidant capacity changes after post-harvest treatment and heat stress (Figure 1).

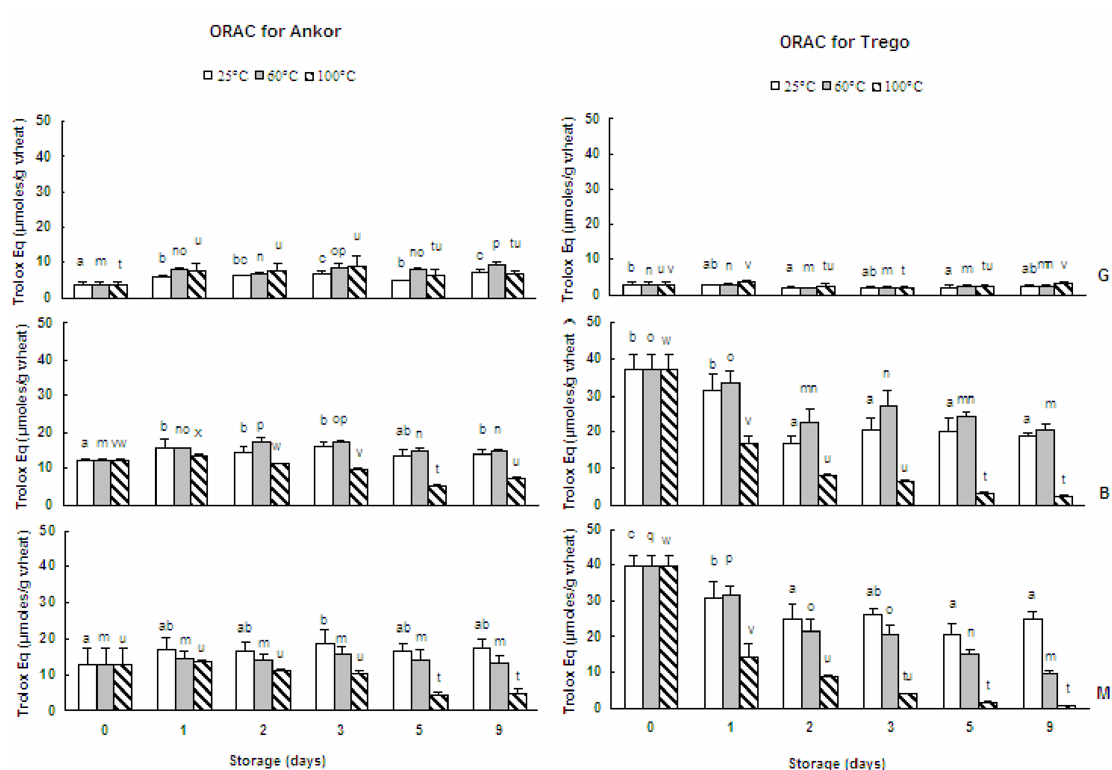

Figure 1. Oxygen radical absorbing capacity of Ankor and Trego wheat during storage. G, B, M stand for grain, bran and micronized bran, respectively. Results are expressed as micromoles of trolox equivalents per gram of wheat. All tests were conducted in triplicate, and mean values are used. Values marked by the same letter are not significantly different ($P < 0.05$).

Table 2. Changes of antioxidant activities in grain, bran and micronized bran of Ankor and Trego wheat between Day 0 and Day 9*.

		Δ ORAC (TE μmoles/g wheat)	Δ ABTS (TE μmoles/g wheat)	Δ % DPPH$^{\bullet}$ quenched	Δ TPC (GA mg/g wheat)
Ankor	Grain	+2.94	-0.07	-3.87	+0.03
	Bran	-4.73	-11.38	+23.51	-0.46
	M-bran	-7.63	-13.18	+34.70	-0.47
Trego	Grain	+0.25	-2.14	-4.38	-0.07
	Bran	-34.69	-7.52	+21.75	-0.34
	M-bran	-38.87	-8.12	+27.52	-0.32

* Trego and Ankor are two hard winter wheat varieties. Δ ORAC, Δ ABTS, Δ % DPPH$^{\bullet}$ quenched, and Δ TPC represent the differences of ORAC, ABTS$^{\bullet+}$, % DPPH$^{\bullet}$ quenched, and TPC in the corresponding wheat sample kept at 100 °C between Day 0 and Day 9. The plus sign (+) indicates an increase for the antioxidant activity, and the minus sign (-) indicates a decrease of the activity except for % DPPH$^{\bullet}$ quenched. The M-bran stands for micronized bran.

ABTS$^{\bullet+}$ radical scavenging activity. 100% ethanol extracts of grain, bran, and micronized bran from Ankor and Trego wheat were examined and compared for their radical scavenging activities against ABTS$^{\bullet+}$. All extracts without heat treatment showed ABTS$^{\bullet+}$ scavenging capacity from 4.39 to 14.39 μmoles of Trolox equivalent (TE)/g of wheat (Table 1). Ankor bran had the highest ABTS$^{\bullet+}$

scavenging capacity, while Trego grain had the lowest under the experimental conditions (Table 1).

Ankor and Trego wheat samples were also investigated for $ABTS^{\bullet+}$ scavenging activity at 25, 60, 100°C for 9 days. The changes in $ABTS^{\bullet+}$ values of wheat samples subjected to heat treatments for several days were shown in Figure 2 (Figure 2). There were no significant changes of $ABTS^{\bullet+}$ values for the grain samples during the nine days of different heat treatments. Bran and micronized bran stored at 100°C exhibited a reduction in their antioxidant activities during nine days (Figure 2). In addition, micronized bran had significantly higher difference of $ABTS^{\bullet+}$ values between day 0 and day 9 compared to bran and grain (Table 2).

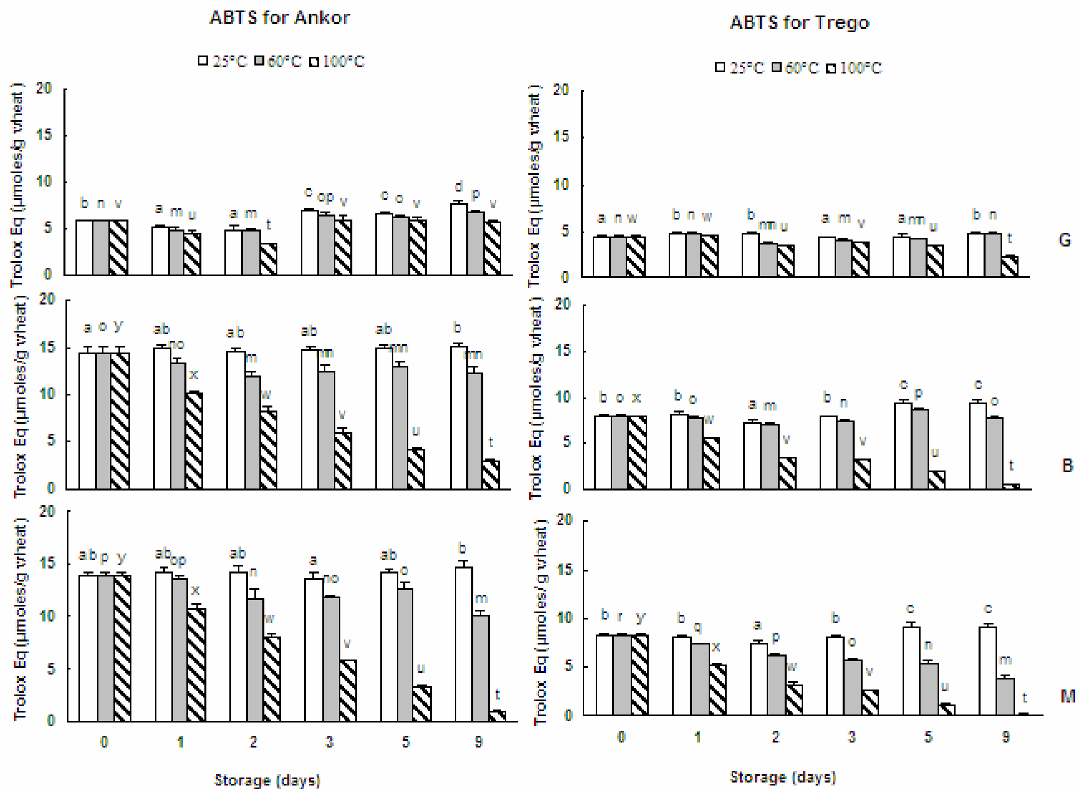

Figure 2. ABTS$^{\bullet+}$ radical scavenging properties of Ankor and Trego wheat during storage. G, B, M stand for grain, bran and micronized bran, respectively. Results are expressed as micromoles of trolox equivalents per gram of wheat. All tests were conducted in triplicate, and mean values are used. Values marked by the same letter are not significantly different ($P < 0.05$).

DPPH$^{\bullet}$ radical scavenging activity. The 100% ethanol extracts of Ankor and Trego wheat samples were analyzed and compared for their free radical scavenging activity against the stable radical DPPH$^{\bullet}$. All wheat extracts, at a concentration of 50 mg/mL, were capable to directly react with and quench DPPH$^{\bullet}$ (Table 1). The % DPPH$^{\bullet}$ remaining was determined at 5 min of each reaction.

Without heat treatment, 100% ethanol extract of Ankor bran quenched 64.4% DPPH$^{\bullet}$ and was highest among all samples on a per weight basis (Table 1). In both varieties, the bran had stronger DPPH$^{\bullet}$ scavenging activity compared to the grain, indicating that radical scavengers are concentrated in wheat bran.

Radical DPPH$^{\bullet}$ scavenging activity of Ankor and Trego stored at 25, 60 and 100°C for nine days is shown in Figure 3 (Figure 3). No obvious differences were seen in DPPH$^{\bullet}$ scavenging activity of grain samples during different heat treatments. Bran and micronized bran samples stored at 100°C showed decreased DPPH$^{\bullet}$ scavenging abilities from day 0 to day 9 (Figure 3). Wheat samples stored at 25°C didn't have significant change during thermal treatment; however, samples heated at 60°C showed a little increase in DPPH$^{\bullet}$ scavenging activity (Figure 3). These data demonstrate that heat treatment at 100°C decreased DPPH$^{\bullet}$ scavenging activity of ethanol extracts of bran and micronized bran. In addition, micronized bran was more sensitive to 100°C treatment than bran and grain in DPPH$^{\bullet}$ scavenging activity (Table 2).

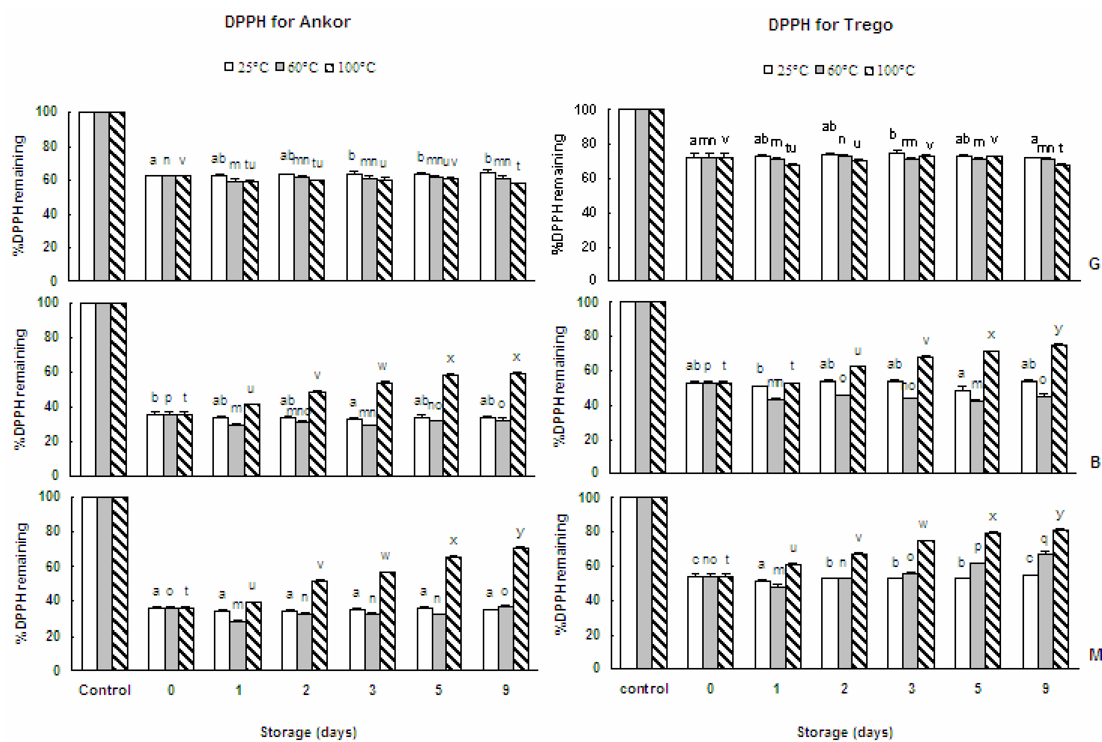

Figure 3. DPPH$^{\bullet}$ radical scavenging activity of Ankor and Trego wheat during storage. G, B, M stand for grain, bran and micronized bran, respectively. The initial DPPH$^{\bullet}$ radical concentration was 100 μM in all reaction mixtures. All tests were conducted in triplicate, and mean values are used. Values marked by the same letter are not significantly different ($P < 0.05$).

Superoxide anion radical ($O_2^{\bullet -}$) scavenging activity. The ESR spectrometry method has been used to measure the presence of free radicals and investigate the radical scavenging properties of antioxidants in natural food (*33, 35*). Superoxide anion radical ($O_2^{\bullet -}$) scavenging activities of Ankor and Trego wheat were evaluated using the 100% ethanol extracts and expressed as % superoxide anion radical ($O_2^{\bullet -}$) remaining. Bran samples showed the greatest superoxide anion

radical ($O_2^{\bullet-}$) scavenging activity, while grain had the lowest activity on a per weight basis (Figure 4).

During the nine days of heat treatment, slight increase in their superoxide anion radical ($O_2^{\bullet-}$) scavenging activities were detected comparing day 0 and day 9 for the grain, bran, or micronized bran heated at 100°C (Figure 4). No significant changes were observed in the samples stored at 25 and 60°C. It needs to be pointed out that the data in Figure 4 were not quantitative.

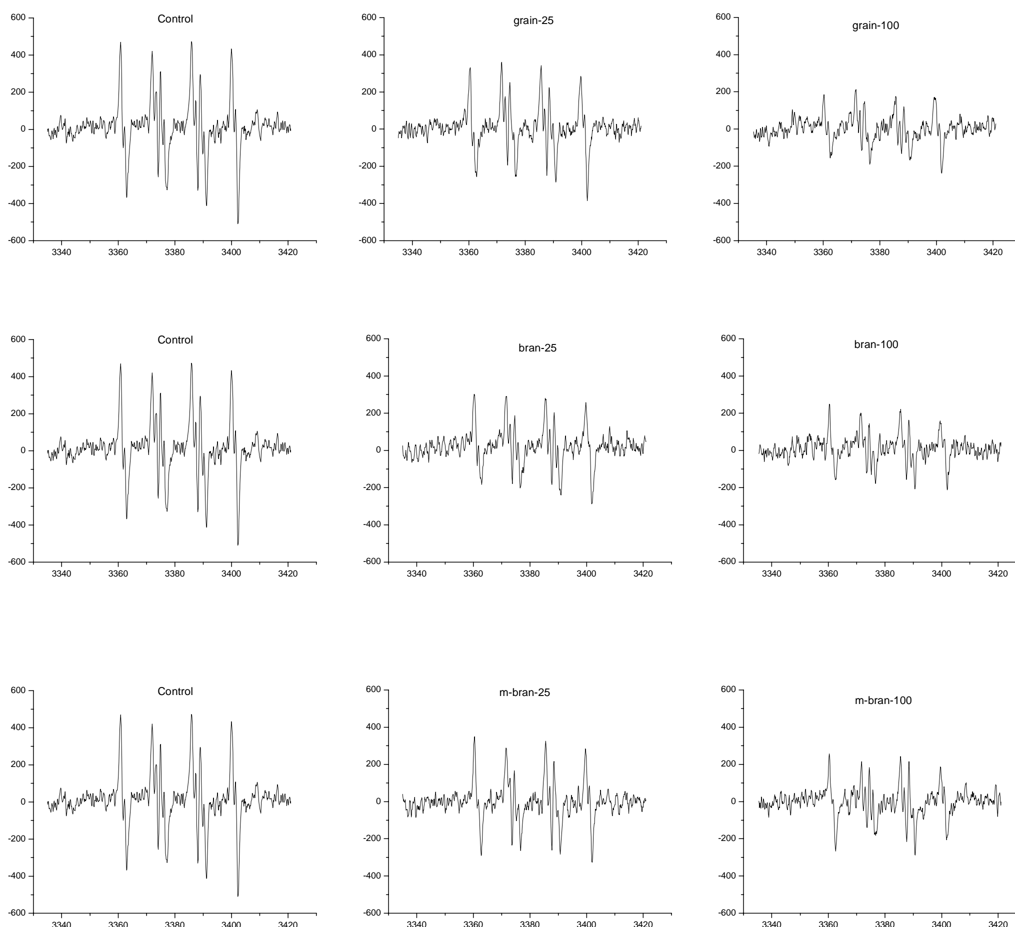

Figure 4. **Superoxide anion radical ($O_2^{\bullet -}$) scavenging activities in Trego grain, bran and micronized bran at Day 0 and Day 9**. Control represents reaction mixtures containing no antioxidants, while grain-25, grain-100, bran-25, bran-100, m-bran-25, m-bran-100 represent Trego grain, bran, and micronized bran samples kept at 25 and 100°C at Day 0 and Day 9, respectively.

Total phenolic content. The Ankor and Trego wheat samples were examined for their total phenolic contents (TPC) expressed as gallic acid equivalent (GE). The greatest TPC of 0.8 mg GE/g wheat was detected in Ankor bran, while

Ankor and Trego grain had the lowest TPC values of 0.36 mg GE/g wheat. In both wheat varieties, bran had higher TPC values compared to grain (Table 1).

Figure 6 shows the changes in the TPC values of wheat samples for nine days. There were no significant changes of TPC values in grain samples of both Ankor and Trego wheat (Figure 6). Bran and micronized bran samples stored at 100°C exhibited a reduction in their TPC values, and micronized bran was more sensitive to 100°C heat treatment than bran and grain comparing difference of TPC values between day 0 and day 9 (Table 2).

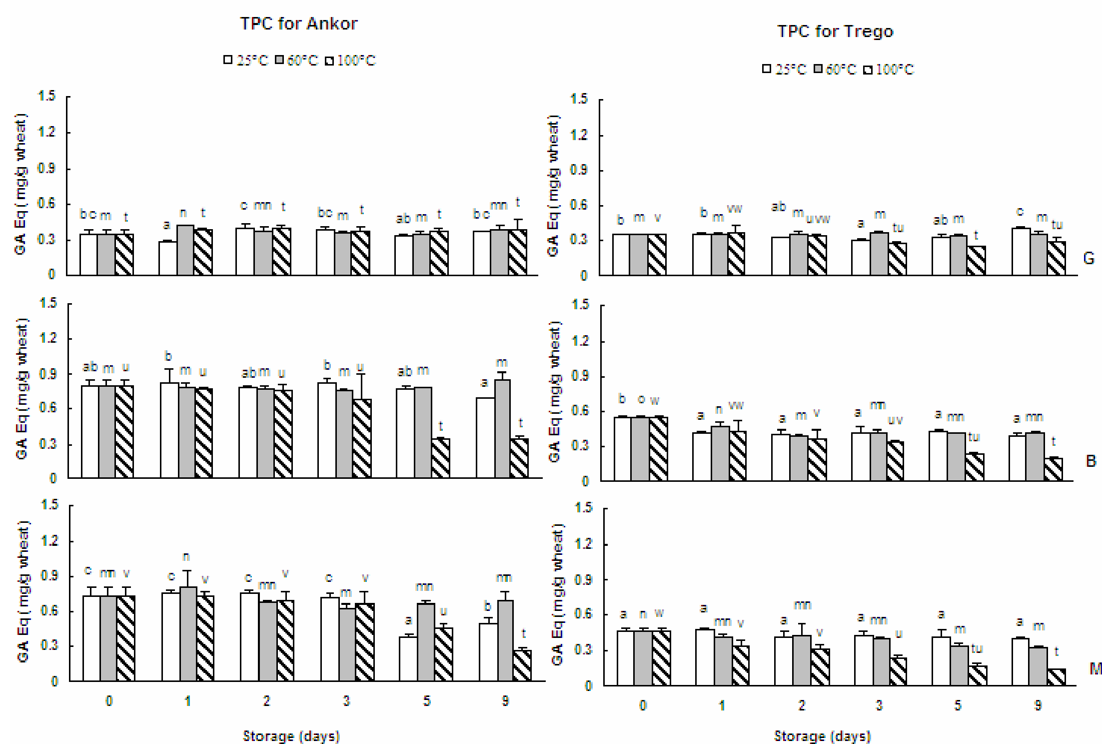

Figure 5. **Total phenolic contents of Ankor and Trego wheat during storage**. G, B, M stand for grain, bran and micronized bran, respectively. Results are expressed as milligrams of gallic acid equivalents per gram of wheat. All tests were conducted in triplicate, and mean values are used. Values marked by the same letter are not significantly different ($P < 0.05$).

Phenolic acid composition. Four phenolic acids, including ferulic, syringic, vanillic, and *p*-coumaric acids, were detected in the wheat extracts. Ferulic acid was the predominant phenolic acid in all extracts. Bran samples had the greatest concentration of ferulic acid, suggesting that ferulic acid and other phenolic acids are concentrated in the bran fraction of Ankor and Trego wheat (Table 3). Phenolic

acid compositions in grain, bran and micronized bran from both Ankor and Trego wheat showed increase in phenolic acid compositions from day 0 to day 9 at 100°C temperature demonstrating that heat treatment may enhance phenolic acid compositions of wheat (Table 3).

Table 3. **Phenolic acid compositions of grain, bran and micronized bran of Ankor wheat stored at 100 °C for Day 0 and Day 9.**

µg/g wheat	Wheat fractions	Day 0	Day 9
vanillic acid	grain	0.65	1.14
	bran	2.10	3.90
	M-bran	2.10	4.16
ferulic acid	grain	4.98	4.29
	bran	11.16	14.66
	M-bran	8.07	9.48
syringic acid	grain	2.86	3.77
	bran	1.87	3.67
	M-bran	1.87	3.16
p-coumaric acid	grain	0.27	0.39
	bran	0.96	1.55
	M-bran	0.96	0.88

Discussion

It is well known that dietary antioxidants can significantly promote human health and prevent chronic diseases by the reaction with reactive oxygen species (ROS) which were produced spontaneously by many biological processes. Epidemiological studies have strongly demonstrated that there is positive correlation between cancer-protective effects and high intakes of vegetables, fruits and whole grains. Additionally, studies have found an inverse relationships in the consumption

of fresh fruits and vegetables with CHD mortality in Great Britain (*36, 37*). In recent years, several epidemiological and clinical studies evaluated the role of antioxidants, primarily vitamin E, in delaying the onset or for treatment of neurodegenerative diseases (*38, 39*). In order to get maximum health benefits from diet, it is important to know the stability of dietary antioxidants in foods after post-harvest treatment. It is generally known that naturally occurring antioxidants could be obviously lost during processing and storage (*40*). In particular, thermal treatments are commonly believed as one of main reasons for the depletion in dietary antioxidants (*20, 21*). This research is part of our continuous effort to determine the effect of post-harvest treatment on the antioxidant stability of wheat.

Both Ankor and Trego wheat samples exhibited significant radical scavenging activities against the peroxyl, $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and $O_2^{\bullet-}$ radicals. The 100% ethanol extracts of Ankor and Trego wheat exhibited ORAC values ranging from 3.07-37.05 μmol of TE/g (Table 1), which is greater, but comparable to those of 2.41-6.25 μmol of TE/g of bran detected in ethanol extracts of Trego and Akron wheat bran (*30*). The $ABTS^{\bullet+}$ radical scavenging capacities of both wheat varieties were comparable to those previously detected in wheat grain and bran (*13, 15-16*). The total phenolic content (TPC) and scavenging capacities against $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and peroxyl radicals of each wheat fraction were also correlated (Table 1, Figure 1, 2, 3, 5). The TPC values of Ankor and Trego wheat ranged from 0.36-0.80 gallic acid equivalents mg/g wheat (Table 1), which are slightly lower than 1.8-4.04 gallic acid equivalents mg/g for Swiss red wheat (*15*). These data suggested that Ankor

and Trego might not differ from previously evaluated hard and soft wheat in their antioxidant capacities.

Grain, bran and micronized bran samples exhibited different stabilities of ORAC scavenging activities under heat stress (Figure 1). Grain samples heated at all temperatures did not show significant change of ORAC values during thermal treatment, which may be due to the hard surface of the grain which was the only area exposed to the heat air. This finding is consistent with one previous study (*24*) that demonstrated wheat kernel inclines to show strong resistance to oxidation because of its morphological character. The ORAC values of the bran and micronized bran stored at 100 °C decreased from day 1 to day 9. Furthermore, micronized bran showed more decrease of ORAC values than bran comparing difference of ORAC values between day 0 and day 9, which demonstrated that micronized bran was more sensitive to thermal treatment throughout the test (Table 2). Smaller particle size and larger exposed surface area may be the reason for the reduction in the thermal stability of wheat antioxidants in the micronized bran compared to bran. However, Zhou et al. (*15*) observed that reduction of particle size of wheat aleurone, a fraction of wheat bran, enhanced the antioxidant availability. The increased antioxidant activities were explained by the increasing of particle surface and the release of antioxidants during extraction. In summary, reduction of particle size may increase the availability of wheat antioxidants from bran, while decrease its thermal stability.

Among the three studied temperatures, only 100°C has decreasing effect of antioxidant abilities on the bran and micronized bran compared with 25°C. For bran stored at 60°C, there was a slight increase in the antioxidant activity observed in both Ankor and Trego, which suggested that moderate thermal treatment may enhance the antioxidant stability of wheat-based food products. Some previous studies proposed that mild heat treatment may produce Maillard reaction products (MRP), which has antioxidant activity and could act as antioxidants in chain breaking and oxygen scavenging activities (*27, 29, 41-44*). This change also might be explained by the enhanced release of antioxidants from the wheat bran by moderate thermal condition, which makes wheat antioxidants more accessible and extractable during the extraction. In brief, higher temperature (100°C) has decreasing effect on the antioxidant activities of bran and micronized bran; while moderate temperature (60°C) has a slight increasing effect on the antioxidant capacities of bran.

As observed in ORAC assay, grain, bran and micronized bran of both wheat varieties all showed similar changes in ABTS$^{\bullet+}$, DPPH$^{\bullet}$ scavenging activities and TPC following post-harvest treatment and heat stress, which suggest that there is positive correlation between different antioxidant property tests (Figures 2, 3, 5, Table 2). Some chemical reactions such as thermal degradation, oxidation and Maillard reactions may explain the change of antioxidant composition and free radical scavenging activity in wheat grain and its fractions (*42-44*). Whole Grain is a preferred form for wheat antioxidant preservation. Reduction of particle size of

bran may increase the effective extractable surface and releasable wheat antioxidants, and decrease antioxidant stability during storage under thermal treatment.

Previous phytochemical studies demonstrated that phenolic acids, tocopherol and carotenoids are the primary natural antioxidants present in wheat grain and bran (*45*). In this study, phenolic acids in grain, bran, and micronized bran of Ankor and Trego wheat were determined using HPLC method. Four phenolic acids, including ferulic, syringic, vanillic and *p*-coumaric acids, were detected in the ethanol extracts of all wheat samples. Phenolic acid compositions were observed to increase in grain, bran and micronized bran from Ankor and Trego wheat at storage temperatures 25°C and 100°C (Table 3). These data demonstrate that heating may maintain or even increase the phenolic acid compositions of wheat, which is consistent with previous studies (*43-44*). Possible explanation may be Maillard reaction products or some conjugated polyphenolics were degraded to extractable single phenolic acids at high temperatures.

In this study, carotenoids and tocopherols were also examined (data not shown). However, there is no acceptable data obtained because the amount of these compounds in the concentrated extracts was under the detection limit. It needs further work for determining the exact chemical composition attributed to the loss of antioxidants.

Ankor and Trego are red and white wheat varieties, respectively. Ankor had significantly higher antioxidant capacities than Trego under both untreated and

treated conditions. However, both varieties showed similar antioxidant activity changes in grain, bran, and micronized bran following the same heat treatments. These results demonstrated that antioxidant stabilities of different wheat varieties were similar following post-harvest treatment and heat stress.

Conclusion

From this study, whole grain has demonstrated to be a preferred storage form for preserving natural antioxidants compared to its fractions. The results from the present study also showed that reduction of particle size may increase the releasable amount of wheat antioxidants from bran, but also decrease the stability of wheat antioxidants during storage and thermal treatment. Some chemical reactions such as oxidation, thermal degradation, and Maillard reactions may contribute to the overall effects of post-harvest treatment and heat stress on the wheat antioxidant properties.

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