ABSTRACT

Title of Document: Enhancing the availability of natural antioxidants in wheat-based food ingredients and food products through improved post-harvest treatments and processing conditions.

Jeffrey Calvin Moore, Doctor of Philosophy, 2007

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Wheat grain has significant antioxidant contents concentrated in the bran fraction, most of which may not be bioavailable in humans because they are not released from matrix materials during digestion. The present study developed solid-state enzymatic and yeast post-harvest treatments, and investigated the effects of these treatments and food processing on the extractable antioxidant properties of whole-wheat based food ingredients and food products. Antioxidant properties investigated in this study included scavenging capacities against cation ABTS radicals, peroxyl radicals (ORAC), hydroxyl radicals, and DPPH radicals, and total phenolic contents and phenolic acid compositions.

The first part of this research developed and validated a high-throughput fluorometric hydroxyl radical scavenging capacity (HOSC) assay. The HOSC assay utilized a Fe(III)/H_2O_2 Fenton-like reaction to generate hydroxyl radicals, fluorescein as detector probe, trolox as an antioxidant standard, and area under the curve measurements to quantify scavenging capacity. The hydroxyl radical purity and potential solvent interference in the assay system were evaluated using electron spin

resonance. The HOSC assay was found to have acceptable performance characteristics including linear range, accuracy, and reproducibility.

The second part of this study investigated the potential of solid-state enzyme and yeast treatments to improve wheat bran antioxidant properties. Both enzyme and yeast treatments were capable of increasing available wheat bran antioxidant properties. Reaction parameters found to influence the effectiveness of these treatments to enhance wheat bran antioxidant properties included enzyme preparation and reaction moisture content for enzyme treatments, and yeast preparation along with dose and treatment time for yeast treatments.

The final part of this research evaluated the effects of processing conditions including bran particle size, fermentation time, and baking conditions on the antioxidant properties of a whole-wheat pizza crust. Baking increased extractable antioxidant properties up to 82%. Fermentation time caused some significant increases, while bran particle size had no influence on extractable whole-wheat pizza crust antioxidant properties.

This study suggests that post-harvest treatment of wheat bran and optimized processing conditions for whole-wheat food products are potential approaches for increasing their extractable antioxidant properties.

ENHANCING THE AVAILABILITY OF NATURAL ANTIOXIDANTS IN WHEAT-BASED FOOD INGREDIENTS AND FOOD PRODUCTS THROUGH IMPROVED POST-HARVEST TREATMENTS AND PROCESSING CONDITIONS.

By

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Dedication

In memory of my grandfathers, Chester Walkowicz and Robert Moore.

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Introduction

Wheat is a global dietary staple. Its use as whole-grain wheat flour has been linked in numerous epidemiological studies to reduced risks for several chronic diseases. Antioxidants compounds present in wheat are thought to play a role in these health promoting properties. Phenolic acids are one of the primary groups of compounds thought to contribute for wheat's antioxidant properties. These phenolic compounds have been characterized in wheat and its milling fractions and found to be primarily concentrated in the bran fraction. The bioavailability of wheat phenolic acids is minimal in humans due to their primarily insoluble bound state in wheat, ester-linked to wheat bran aleurone cell wall materials. These ester-linkages are not hydrolyzed by human digestive enzymes, inhibiting their availability for absorption in the in the small intestine.

Increasing the bioavailable antioxidant properties of wheat based food products is one potential approach for improving their health promoting properties. No studies to date have investigated the effectiveness of solid-state enzyme or yeast fermentation treatments to improve the antioxidant properties of wheat bran. In addition, no studies to date have evaluated the effects of processing conditions on the antioxidant properties of whole-wheat pizza crust, a commonly consumed wheat-based food product.

The overlying goal of this research is to increase the antioxidant properties of wheat and wheat based food products. The primary objectives of this investigation are:

- To develop an *in-vitro* hydroxyl radical scavenging capacity with the following characteristics: high-throughput capacity, generates pure and consistent concentration of hydroxyl radicals, expresses results relative to a standard, evaluated solvent compatibility, and validated performance characteristics including linearity, reproducibility, accuracy, and precision.
- To develop solid-state enzyme reaction systems for wheat bran, and investigate their potential for improving wheat bran antioxidant properties using Viscozyme L, Pectinex 3XL, Ultraflo L, Flavourzyme 500L, Celluclast 1.5L, and porcine liver esterase enzyme preparations.
- To develop solid-state yeast fermentation reaction systems for wheat bran, and investigate their potential for improving wheat bran antioxidant properties using three commercially available yeast preparations.
- To evaluate the effects of processing conditions including bran particle size, dough fermentation time, and baking time and temperature, on the antioxidant properties of whole-wheat pizza crust.

Chapter 1: Literature Review

1.1 Wheat's Importance and Classifications

Wheat (*Triticum spp.)* has historically been a global dietary staple. It has been credited along with other cereal crops with fueling the rise of civilization. The origins of wheat are thought to date back more than 10,000 years to the Fertile Crescent region in the Middle East where remains of the wild progenitors of wheat have been discovered (*1*). Today wheat is grown on the most land area of any commercial crop (*2, 3*). It is also the most produced food crop globally, with 420 million metric tons produced for food in 2003 (*2, 3*). Wheat in the form of bread currently and historically has provided more nutrients than any other food source world-wide (*4*). Countries currently producing the most wheat include China, European Union, India, and the United States (*5*).

Wheat is generally categorized into six classes according to milling and end-use attributes such as grain hardness and color, as well as growing season (*4*). Durum wheat (*Triticum durum*) which comprises approximately five percent of global wheat production is the hardest type of wheat and is predominantly used in the production of pastas (*2*). The remaining ninety-five percent of wheat produced is referred to as common wheat (*Triticum aestivum*). Common wheat includes five classes of wheat, hard red winter wheat, hard red spring wheat, soft red winter wheat, soft white wheat, and hard white wheat – the newest class of wheat grown in the U.S. (*2, 6*). The

primary factor determining the suitability of common wheats for specific food applications are their gluten contents. Gluten is a complex mixture of proteins in wheat that gives wheat doughs their unique viscoelastic properties commonly referred to as dough strength (*4*). Hard wheat has a high gluten protein content compared to other wheat classes. This high gluten content produces higher viscoelastic properties in doughs and makes hard most suitable for products such as bread and pizza crust. Soft wheat has low gluten protein content and is used for production of cakes, cookies, and crackers (*4*). With bread as a predominant source of nutrients world-wide, hard wheat is the most produced type of common wheat, accounting for more than 65% of wheat production the US (*4, 7*). In addition to bread, recent consumption trends in the US have shifted some hard wheat usage to other products such as pizza (*7*).

While the global demand for wheat is expected to increase, wheat production in the United States (U.S.) as of 2005 has faced several challenges and is projected to experience only modest gains in demand due to several factors (*8*). Planted area in the US for wheat in 2005 has dropped nearly 30 percent since the 1980's because of lower returns relative to competitive crops such as soybeans and maize which have seen increased yields from genetic improvements and changing government programs (*5, 8*). In addition, changing consumer diet trends with lower carbohydrate consumption dropped per capita consumption of wheat in 2004 to the lowest levels since the 1980's (*5*). The US share of the world wheat trade has been decreasing due to increased global competition, and is projected to increase slightly from 2005 to 2015 (*8*).

The challenging situation for wheat in the US as of 2005 has generated considerable interest in research to improve the competitiveness and farm-gate value of wheat (*5*). Most current research has focused on improving the yield, disease, pest, drought resistance, and the functional properties of wheat through genetic modifications. Little research, however, has focused on the potential of improving the nutritional or health promoting properties of wheat.

1.2 Wheat's Fractions and Their Nutritional Properties

Common wheat consists of three primary milling fractions: germ, endosperm, and bran. The majority of the wheat kernel (the caryopsis) is the endosperm and consists of mostly one type of cell specialized for reserves of starch and storage proteins (*9*). These stores are the major source of energy during germination for the embryo (*9*).

The germ and bran together provide the majority of the bioactive compounds in the wheat grain (*9*). Germ represents the smallest portion (2-3% by weight) of the wheat grain and consists of the embryonic axis and scutellum. It contains lipids, and small amounts of protein and minerals. It also contains bioactives including B-vitamins and vitamin E in the lipid fractions, along with phytin, phytosterols, and phenolic acids (*9,10*). The physiological function of the germ is in developing new embryonic plants and absorbing nutrients during germination (*9*).

Wheat bran is the outer most fraction of the wheat kernel comprising 14-16% of the kernel by weight, and consists of multiple layers. Aleurone is the inner most bran layer consisting of a single layer of cells adjacent to the endosperm. Moving

outward towards the periphery of the wheat kernel are the hyaline layer (nucellar epidermis), seed coat (testa), inner pericarp (cross and tube cells), and outer pericarp (beeswing bran). Aleurone cells contain high levels of fiber, small levels of lipids, and protein with high levels of lysine and arginine amino acids (*9*). Of all the layers of wheat, aleurone has the highest concentration of vitamins, minerals, and phytochemicals including phytin crystals and associated minerals, B-vitamins niacin and thiamine, tocopherols and tocotrienols (tocols), and phenolic acids (*9*). The aleurone layer has physiological functions in wheat including protective roles against attacks by bacterial, fungal, and insect pests. Additional functions of aleurone include controlling hydration processes and activating hydrolytic enzymes during germination (*Fulcher 2002*). Other layers within the bran fraction include the pericarp, testa, and nucellar epidermis. These layers contain similar bioactives as aleurone, but in lower concentrations, and participate in similar physiological functions (*9*).

The high levels of bioactive compounds found in wheat bran and germ fractions have promoted the consumption of whole-wheat food products as opposed to refined wheat products which do not use these bioactive rich wheat fractions.

1.3 Wheat and Evidence of its Health Promoting Properties

The widespread human consumption of refined wheat products has been a relatively recent event in the history of wheat consumption (Slavin et al 2004, Spiller et al 2002). Refined wheat products have historically been preferred due to their perceived improved quality compared to whole wheat products. Refined wheat was

not available to the majority of the human population until recently due to cost-prohibitive inefficient milling technologies. In 1873, the invention of the roller mill provided a milling technology to efficiently separate wheat fractions (*11,12*). This event made refined wheat products affordable to the majority of the population, and fueled the increased consumption of refined wheat products.

The health promoting aspects of whole-grain foods have been recognized since the 4th century BC (*11*). It was not until the 1970's, however, that researchers attributed these properties to fiber and other bioactive constituents present in wheat bran and germ (*11*). This "fiber hypothesis" was developed from observational studies in the 1970's of African populations. These populations consumed mostly whole plant based diets high in fiber, and were free of many Western diseases such as cardiovascular disease and colon cancer (*13-15*).

Since that time, numerous epidemiological and clinical studies have presented strong evidence that consumption of whole-grain foods significantly reduces the risks for numerous chronic conditions (*11, 13*). A recent overview in 2004 by Slavin (*11*) of the available data for cancer concluded that substantial scientific evidence exists to demonstrate that whole-grain consumption reduces the risk for several types of cancer. This conclusion was drawn based on numerous studies including a recent meta-analysis with 43 of 45 studies showing reduced cancer risk with increased whole-grain consumption (*11, 16, 17*). Additional support for this hypothesis came from a recent systemic review of case-control studies from 1983-1996 in Italy (*11, 16, 17*). Assessments of the available data for cardiovascular disease (CVD) have shown strong scientific evidence to support a reduced risk of this disease with whole-

grain consumption (*11, 18-20*). Specifically, a meta-analysis of twelve studies supported a 26% risk reduction for CVD for regular whole-grain intakes (*18, 19*). Additionally, a more recent epidemiological study found people with higher dietary levels of whole-grains to have a 29% reduced risk of CVD compared to those with low whole-grain consumption (*21*). Significantly reduced risks for type-2 diabetes, ischemic stroke, obesity, and overall incidence of all-cause mortality associated with whole-grain consumption have also been reported (*11, 22, 23*).

Given the significant evidence linking whole-grain consumption to reduced risk of chronic disease, recent research has been aimed to identify mechanisms or bioactive compounds responsible for these health promoting properties. Numerous animal and cohort studies have suggested that the bran fraction of wheat is the key factor responsible for wheat's protective effects against cancer (*24-32*). It was previously thought from the 1970's "fiber hypothesis" that fiber was the active component in wheat bran responsible for these effects. Recent epidemiological studies and a pooled analysis of cohort studies, however, have refuted the role that dietary fiber alone plays in reduced risk of chronic disease (*21, 33-36*). This has indicated that another wheat bran component besides the fiber component is responsible (*21, 33-36*). One hypothesis is that antioxidants, which are known to be present in higher concentrations in wheat bran compared to endosperm, may be a key bioactive factor for this relationship (*11, 37, 38*). A recent animal study by Carter and others (*39*) showed that the *in-vitro* antioxidant potential of wheat brans were correlated with their *in-vivo* antitumor activities. *Overall, the hypothesis that antioxidants in wheat are responsible for its health promoting properties has*

promoted further research in this area.

1.4 Free Radicals, Oxidative Stress, and Human Health

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are a diverse group of reactive species known to be generated *in-vivo*. ROS and RNS include the superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$), peroxyl radicals ($ROO^{\bullet}$), singlet oxygen ($^1\Delta gO_2$), nitric oxide ($^{\bullet}NO$), nitrogen dioxide ($^{\bullet}NO_2$), and peroxynitrite ($OONO^-$). Most of these species are free radicals. These chemical species have an unpaired electron in their outer valence shell which makes them reactive. $^1\Delta gO_2$, in contrast, is an energetically excited state of dioxygen (O_2). The generation of $O_2^{\bullet-}$ is known to occur in biological systems as a result of electron leakage from the mitochondrial electron transport chain used in aerobic respiration (*40-43*). Generation of other free radicals *in-vivo* is thought to occur chemically or enzymatically through a variety of mechanisms often involving other ROS or redox reactive transition metals. Singlet oxygen is generated through the photosensitization processes (*44-47*). ROS and RNS are capable of reacting with and damaging biomolecules including lipids, proteins, nucleic acids. The generation of ROS or RNS that is not balanced *in-vivo* by defense systems leads to what has been termed 'oxidative stress' (*46*). It is thought that *in-vivo* oxidative stress plays a role in human health.

One of the first theories linking free radicals or oxidative stress to human health was the "free radical theory of aging" put forth by Denham Harman in 1956 (*48*). Harman suggested that oxygen centered free radicals produced *in-vivo* from

redox chemistry during aerobic metabolism cause cumulative oxidative cellular damage leading to aging and eventually death (*48, 49*). Harman also found the outcomes of ionizing radiation similar to those of aging including cellular damage, mutagenesis, and cancer (*49*). Interestingly, the generation of hydroxyl radicals from water using ionizing radiation was described not long before Harman's theory in 1948 (*50*). Since that time, evidence has continued to support Harman's theory (*51*). This has included evidence of increased generation of oxidative species, decreased antioxidant capacity, or reduced oxidative damage repair, all seen concurrent with aging in animal and cell studies (*51*). As a result, the cumulative oxidative damage theory remains today one of the leading mechanistic theories to explain the aging process (*49, 51, 52*).

In addition to the aging process, oxidative stress from reactive oxygen and nitrogen species have been implicated in numerous human health conditions. The involvement of oxidative stress in the pathogenesis of CVD, the leading cause of death in the US and Europe, has been extensively studied (*53*). Oxidative stress may be involved in the oxidative modification of low density-lipoprotein (LDL). This modification of LDL increases its affinity for macrophages and leads to lipid-laden foam cell formation, a key step in the development of atherosclerosis (*53-55*). In addition, oxidative stress has been attributed as a predominate cause of myocardial damage following ischemia (post-ischemia reperfusion) (*56, 57*). Oxidative stress has also been linked to cancer, the second leading cause of death in the US. Potential mechanisms linking cancer and oxidative stress include oxidative modification of DNA, initiation of inflammatory responses, inhibition of gap junction intercellular

communication, activation of matrix metalloproteinases, and activation of nuclear factor kappa B (NF-kappa B) and activator protein (AP-1) signal transduction pathways (*58, 59*). Development of senile plaques, the hallmark pathological lesions associated with Alzheimer's disease has been associated with oxidative stress through deposition of β-amyloid proteins (*60, 61*). Additional chronic conditions in which oxidative stress have been implicated include Parkinson's disease (*62*), inflammation (*63*), and diabetes (*64*). *Overall, the potential mechanisms through which ROS and oxidative stress may be involved in the etiology of chronic conditions include: alteration of biomolecules causing oxidative modification of proteins (65), oxidation of lipids (66), strand breaks and modification to nucleic acids (67), modulation of gene expression through activation of redox-sensitive transcription factors (68, 69), and modulation of inflammatory responses through signal transduction (63).*

1.5 Antioxidants and Human Health

Given the numerous lines of evidence linking oxidative stress and ROS or RNS to chronic conditions, there has been tremendous research interest in the role of dietary antioxidants in preventing these conditions. A simple and broadly accepted definition of an antioxidants described by Halliwell is "a molecule which, when present in small concentrations compared to that of an oxidizable substrate, significantly delays or prevents the oxidation of that substrate" (*70*). *In-vivo*, antioxidants can function through at least three processes. Firstly they can directly react with or scavenge free radicals. Mechanisms for free radical scavenging include electron transfer, hydrogen atom transfer, or sequential proton loss electron transfer

(*71*). Secondly, antioxidants can induce antioxidative enzyme systems such as superoxide dismutases which scavenge superoxide anion radicals, or inhibit oxidative enzymes such as cylcooxygenases which can generate ROS (72). Thirdly, antioxidants can form thermodynamically stable chelate complexes with redox active transition metals such as iron and copper. These chelate complexes can prevent ROS generation by limiting access of oxygen and H_2O_2 to metal coordination sites, thereby rendering them reactively inert (*73*).

One of the origins of the 'antioxidant hypothesis' came from a report by Gey in 1987 which inversely related plasma concentrations of vitamins C and E and β-carotene in European men to ischemic heart disease mortality rates (*74, 75*). Gey also acknowledged in this report the importance of synergistic interactions between antioxidants to provide health benefits (*74, 75*). Since that time, the role of these dietary antioxidative vitamins in reducing risk for CVD and cancer has been further investigated through *in-vitro*, animal, epidemiological, and clinical studies. In relation to CVD, numerous *ex-vivo*, animal, and epidemiological studies have reported that vitamin E may significantly reduced the risk for this disease (*53, 76*). A famous clinical study, The Cambridge Heart Antioxidant Study, evaluated 2000 patients and found that vitamin E supplementation reduced risk of myocardial infarction and cardiovascular related mortality by 47% after one year of treatment at 400 or 800 IU/day (*77*). Similar recent clinical studies, however, have not supported this correlation (*78, 79*). In relation to cancer, intake of vitamin C or lycopene has been negatively correlated in some epidemiological studies (*53*). Risk of Alzheimer's disease has been reportedly decreased by high dietary intakes of vitamins C and E in

some cohort studies (*80*). Overall, while some lines of evidence from *in-vitro*, *ex-vivo*, and animal studies have correlated intake of pure antioxidant compounds such as vitamins E and C, and B-carotene to reduced chronic disease risk, clinical intervention studies have not consistently supported these effects (*53, 79, 81-83)*.

The strongest evidence linking reduced risks for chronic diseases to antioxidants have come from studies involving consumption of foods naturally rich in numerous antioxidants s opposed to single purified vitamins with antioxidant properties (*53, 83, 84*). This has included foods such as fruits, vegetable, and whole-grains a (*53, 83, 84*). A recent review of clinical studies compared consumption of tomatoes versus lycopene in reducing biomarkers of carcinogenesis in humans (*85*). This study found tomato products rich in lycopene, but not just lycopene to be effective in reducing these biomarkers (*85*). A cohort study by Dai and others (*86*) reported that consumption of antioxidant rich fruit and vegetable juices but not their constituent antioxidant vitamins E, C, or β-carotene were associated with reduced risk for Alzheimer's disease. Another recent study found that the reduced risk of CVD associated with whole-grains consumption could not be attributed to its vitamin E or B-6 composition (*35*). Together these studies have suggested that antioxidative vitamins in fruits, vegetables, and whole-grains cannot solely explain the health promoting properties of these foods.

Whole-wheat, as a whole-grain, has attracted recent attention for its potential in health promotion given its widespread consumption. This has included changes in the US Dietary Guidelines which now suggest consumption of three to ten servings of whole-grains per day (*87*). Recent launches of whole-grain food product lines in the

US have indicated improved consumer interest in these food products (*88*).

1.6 The Antioxidant Contents of Wheat

Studies on antioxidants in wheat have investigated its antioxidant composition, as well as its antioxidant properties such as free radical scavenging and chelating properties. It is known today that wheat naturally contains numerous classes of antioxidant compounds including phenolic acids, alk(en)ylresorcinols, phytic acid, steryl ferulates, plant lignans, carotenoids, tocopherols, and tocotrienols (*89-103*).

Although an understanding of the antioxidant properties of wheat bioactive compounds did not occur until later, actual identification of some of these compounds in wheat dates back to the early twentieth century. Vitamin E was discovered in wheat in the 1920's, and its antioxidant properties in wheat germ were studied in 1936 in lipid systems (*104, 105*). Work almost 50 years later identified several vitamers of vitamin E in wheat (*106*). More recent studies have confirmed the presence of α-, β-, δ-, and γ-tocopherols and α-T3 and β -T3 tocotrienols and found the total concentration of tocols in soft wheat to be approximately 75 mg/kg dry weight (*89, 93, 97*).

The presence of carotenoids in wheat was reported in the early 20th century, but it was debated early on if these carotenoids had provitamin A activity (*107*). A study by Bowden and Moore in 1933 found xanthophylls, a group on carotenoids lacking provitamin A activity, to be a major type of carotenoids in wheat concentrated in the germ fraction (*108*). More recent studies have confirmed this, showing the

predominate carotenoids present in wheat grain to be lutein and zeaxanthin with concentrations of 0.5-144 μg/g and 2.2-27 μg/g grain respectively (*89, 93, 95, 109*). Carotenoids with provitamin A activity are present in wheat grain in small concentrations including β-cryptoxanthin at 0.18-13μg/g, and β-carotene at 0.09-0.40 μg/g (*89, 93, 95, 109*). Zhou and others (*92*) examined the carotenoid contents of two varieties of hard red wheat bran, and found lutein to be the predominant carotenoid with concentrations ranging from 0.97 to 1.43 μg/g wheat bran. This report also found smaller concentrations of β-carotene, zeaxanthin, and β-cryptoxanthin ranging from 0.03 to 0.40 μg/g wheat bran (*92*). A report by Adom and others (*96*) evaluated carotenoid concentrations in different wheat fractions. They found 12-, 4-, and 2-fold higher concentrations of zeaxanthin, lutein, and β-cryptoxanthin respectively in the bran/germ fraction of wheat compared to endosperm. Together, these studies have shown wheat to contain low amounts of antioxidative carotenoids, mostly concentrated in the germ and bran fractions.

Secoisolariciresinol diglycoside is a diphenolic lignan compound converted to the mammalian lignans enterodiol and enterloactone by intestinal microflora. It has been detected in wheat bran, but in relatively low concentrations at 1.1 μg/g (*101, 110*). Steryl ferulates are ferulic acid esters of plant sterols. They have been detected in wheat bran in concentrations ranging from 30-39 mg/100g (*98*). Phytic acid has been reported in whole wheat flour in the range of 8.5 mg/g (*111*). Alk(en)ylresorcinols are compounds containing long nonisoprenoid side chains attached to phenolic acids. They have been detected in wheat bran with total concentrations of 3.2g/kg (*103*). Although present in wheat, lignans, steryl ferulates,

phytic acid, and alk(en)ylresorcinols are not thought to be the predominate compounds responsible for its antioxidant properties.

Phenolic acids are hydroxylated derivatives of benzoic and cinnamic acids and in wheat include including vanillic, syringic, *p*-hydroxybenzoic, gallic, *p*-coumaric, caffeic, ferulic, sinapic acids, and dehydrodimers of ferulic acid (*93, 112*). They are thought to one of the primary groups of compounds responsible for the total antioxidant and health promoting properties of wheat (*20, 37*). The presence of ferulic acid in its free and protein-bound states in wheat has been known since the 1960's from the studies of el-Basyouni and Towers (*113*) and Fausch and others (*114*). Gallus and Jennings in 1971 found the majority of wheat phenolics in an ester bound state, and Fulcher and others studying the localization of wheat phenolics found ferulic acid to be most concentrated in the aleurone layer of wheat bran (*115, 116*). It is now known that phenolic acids exist in wheat in three primary states, soluble free, soluble conjugated, and insoluble bound. A recent study by Moore and others (2005) found the total phenolic content of soft wheat grains including soluble free, soluble conjugated, and insoluble bound fractions measured with HPLC with UV/vis detection to range from 455 to 621 μg/g in wheat grain (*93*). This study also found the insoluble bound fraction to comprise the majority (91%) of wheat phenolics, with soluble conjugated and soluble free fractions comprising 8.7% and 0.58%, respectively (*93*). Ferulic acid is the primary phenolic acid in wheat grain with total concentrations (soluble free, soluble conjugated, and insoluble bound) of 455-607μg/g. Smaller concentrations of *p*-hydroxybenzoic, vanillic, syringic, *o*-coumaric, *p*-coumaric, salicylic, sinapic acids are also present in wheat. The

individual concentrations of these phenolic acids range from 2-14 μg/g (*90, 93, 100, 117, 118*). Phenolics have been found to be most concentrated in the bran fraction, specifically the aleurone layer, with total ferulic acid concentrations ranging from 89 to 2020 μg/g bran (*89-92, 96, 100, 119, 120*).

Besides direct quantification of the individual antioxidant contents of wheat, indirect methods have also been used to determine the total concentration of certain antioxidant classes found in wheat such as total phenolic contents and total flavonoid contents. Several studies have evaluated the total phenolic contents (TPC) of wheat and its fractions using the Folin-Ciocalteu reagent. Reported TPC values for wheat grains have ranged from 0.23 to 9.28 mg gallic acid equivalents (GAE) per gram, while wheat bran, germ, and wheat based food product TPC values have been reported to ranged from 2.2-3.5, 4.4-6.1, and 0.2-1.2 mg GAE/g respectively (*89-93, 95, 96, 117, 121-127*). Other studies using ferulic acid as a standard have reported TPC value ranges for wheat grain, endosperm, bran, and germ of 0.35-2.06, 0.23-0.82, 0.46-6.56, and 3.49-12.18 mg ferulic acid equivalents per gram (*128-130*). The total flavonoid content of wheat grains was evaluated by Adom and others (*94, 95*) and reported to be 0.07-0.17 and 0.96-1.38 μmoles catechin equivalents per gram grain for soluble free and insoluble bound fractions respectively. A later study by this group evaluted flavanoid contents in the endosperm and germ/brans fractions of five wheat varieties. This study found the higher concentrations of flavanoids in the germ/bran fractions (0.740-0.940 μmoles catechin equivalents per gram grain) compared to the endosperm fraction (0.06 to 0.08 μmoles catechin equivalents per gram grain) (*96*).

1.7 The Antioxidant Properties of Wheat

Given the hypothesis that the antioxidant compounds in wheat may be responsible for its health promoting properties, many recent studies have evaluated wheat's antioxidant properties *in-vitro* and *ex-vivo*. These studies have included scavenging capacities against numerous ROS and non-physiologically relevant free radicals, chelating capacities against reactive transition metals, inhibition of lipid oxidation, and reducing capacity. The ABTS cation radical and DPPH radical are two non-physiologically relevant radicals often used to analyze *in-vitro* the antioxidant properties of botanical materials such as wheat. Reports of DPPH radical scavenging capacity for wheat found soft and hard wheat grains to exhibit EC_{50} ranges of 23-27 and 2-5.2 mg wheat equivalent per mol respectively, with EC_{50} representing the concentration of sample necessary to scavenge 50% of DPPH radicals as steady state (*90, 93, 131*). Hard wheat brans and aleurone, however, have shown higher DPPH scavenging capacities (lower EC_{50} value) using this method with a reported range of 6-12 mg/mol (*89, 90*). Cheng and others reported DPPH scavenging capacity ranges for hard wheat grain and bran of 0.9-1.1 and 1.8-3.2 μmoles trolox equivalents per g (μmol trolox/g) using the relative DPPH scavenging capacity assay (RDSC) (*132*). Numerous groups have reported the scavenging capacity of wheat and its fractions against the ABTS cation radical. These results have ranged from 1-36, 5-35, 23-24 μmol TE/g for soft and hard wheat grains, hard wheat brans, and hard wheat aleurones respectively using several ABTS methods (*90-93, 119, 121-123, 130, 133, 134*).

The peroxyl radical is a physiologically relevant radical involved in chain lipid peroxidative reactions. The peroxyl radical scavenging capacity of wheat and its fractions has been reported using several available methods. Oxygen radical absorbance capacity (ORAC) assay results have been reported including 32-51, 15-107, and 125-136 μmol TE/g for soft and hard wheat grains, hard wheat brans, and hard wheat aleurone respectively (*90, 92, 93, 119, 135*). Another method evaluating scavenging capacity against the peroxyl radical is the PSC method. Adom and others using PSC reported the hydrophilic antioxidant capacities of soluble free and insoluble bound wheat antioxidants to be 10.4 and 289 μmoles vitamin C equivalents per 100g wheat respectively (*136*). This same group also reported lipophilic antioxidant capacity of whole grain wheat using PSC as 3.49 μmoles Vitamin E per 100 g wheat. Lastly, Adom and others (*136*) using trolox as a standard found the hydrophilic antioxidants of whole wheat to contribute more than 99% of the total (hydrophilic plus lipophilic) PSC for whole wheat. Two reports by Adom and others utilized the total oxyradical scavenging capacity (TOSC) assay to evaluate the peroxyl radical scavenging capacity of wheat grain samples. These two reports found soluble free and insoluble bound extracts of wheat to exhibit TOSC value ranges of 5-14 and 26-69 respectively (*94, 95*).

Scavenging activities against the physiologically relevant superoxide anion radical, hydroxyl radicals, and hydrogen peroxide have been reported by several groups. Superoxide anion radical ($O_2^{\bullet -}$) scavenging capacity values for wheat have been reported using both absorbance and electron spin resonance (ESR) spectroscopy techniques. Both detection techniques have been coupled with a hypoxanthine-

xanthine oxidase system to generate $O_2^{\bullet-}$. Reports using an absorbance spectrophotometric method with nitroblue tetrazolium (NBT) as a detector probe found Swiss wheat grain, Swiss and hard wheat brans, and Swiss wheat aleurones to scavenge 22, 22-44, and 41-57 % of $O_2^{\bullet-}$, respectively (*90, 119, 133*). Using an ESR spin-trapping assay, Zhou and others (*92*) reported that both Alliance and Witchita varieties of hard red winter wheat bran had $O_2^{\bullet-}$ scavenging capacities of 12.96 and 10.96 units of superoxide dismutase per gram of bran respectively. Martinez-Tome and others (*137*) reported the hydroxyl radical scavenging activities of 200mg/mL extracts of wheat bran and wheat bran powder using the deoxyribose method. Their results ranged from 79 to 96 % inhibition respectively for wheat bran and wheat bran powder compared to 32% inhibition exhibited by a 0.5mM trolox standard. Zhou and others (*92*) reported that extracts of both Alliance and Witchita hard wheat brans exhibited significant hydroxyl radical scavenging using an ESR spin-trapping assay. These results by Zhou and others, however, were qualitative and not quantitative (*90*). Boveris and others (*138*) found wheat bran extracts to inhibit the formation of hydroxyl radicals generated with a NADPH iron-citrate system measured with an ESR DMPO spin trapping assay. Lastly, Martinez-Tome and others reported the ability of wheat bran extracts to scavenge of hydrogen peroxide with results ranging from 60-84% H_2O_2 scavenged (*137*).

The antioxidative chelating properties of wheat extracts against the Fe(II) and Cu(II) ions have been reported by several groups. Fe(II) chelating capacities ranges of 0.1-5.4, 0.4 to 2.0, and 1.25-1.75 mg EDTA equivalents per g wheat for soft and

hard wheat grains, hard wheat bran, and Swiss red wheat aleurone respectively using a spectrophotometric method. (*89-93, 119, 121, 123, 124, 125, 129)*. Evaluating the Cu(II) chelating properties of Alliance and Witchita hard red winter wheat brans with an ESR method, Zhou and others (*92*) found both varieties to form significant chelate complexes for Cu(II) with wheat extract concentrations of 50 mg wheat bran equivalents per mol.

Inhibition of lipid peroxidation by wheat extracts has been evaluated using several systems. Oneneho and Hettiarachchy (*139*) found extracts of durum wheat bran to inhibit the oxidation of soy oil as much as 70% under the Active Oxygen Method conditions measured using the peroxide value method (PV). Boveris and others (*138*) found a wheat bran extract to inhibit the lipid oxidation of rat microsomes with an EC_{50} of 2.7 mg/mol. This study also found the same wheat bran extract to inhibit lipid radical formation measured with ESR spin trapping techniques using a-(4-pyridly 1-oxide)-N-t-butyl nitrone (POBN). Mageed and others (*140*) found extracts of wheat bran to significantly inhibit the oxidation of linoleic acid using the TBARS assay. Two studies by Amarowicz and others found extracts of both wheat grains and embryos to significantly inhibit lipid oxidation in the β-carotene-linoleate system (*126, 141*). Another study evaluated the ability of extracts of soft and hard wheat fractions to inhibit lipid oxidation with this system, and found bran to have the highest activity followed by germ and endosperm with β-carotene retention values (*130*). Martinez-Tome and others (*137*) found wheat bran extracts to significantly inhibit the oxidation of ox brain phospholipids liposomes (68-78% inhibition) using a pH 7.4 buffered system with a Fe(III)/ascorbate ROS generating

system and TBARS analysis. A study by Baublis and others (*142*) found wheat extracts to significantly inhibit the oxidation of a salmon oil emulsion held at 25ºC for 120 hours by measuring TBARS. Another study by Baublis and others (*143*) found wheat bran and grain extracts to inhibit to the iron/ascorbate and peroxyl radical induced oxidation of phosphatidylcholine liposomes measured using TBARS to measure lipid oxidation. Zielinski and others (*134*) demonstrated the ability of wheat grain extracts to inhibit the AAPH-induced lipid oxidation using a unilamellar liposome model. Malecka (*144*) reported that unsaponifiable materials from wheat germ oil were able to significantly inhibit the oxidation of rapeseed oil when added at 0.3%. Krings and others (*145*) reported roasted wheat germ extracts to significantly inhibit the oxidation of stripped corn oil under accelerated oxidation conditions by measuring the formation of diene hydroperoxides. Several studies have investigated the ability of wheat to extend the oxidative stability of lipids by measuring the protection factor (defined as ratio of sample induction time versus control induction time) using an oxidative stability index method (OSI). These studies have reported protection factors of 1.19-2.1 and 1.35-2.1 for hard and soft wheat grains, and hard and soft wheat brans, respectively, using a variety of substrate oils and reaction conditions (*129, 131, 137*).

Several studies have evaluated the capacity of wheat samples to protect biomolecules such as LDL and supercoiled DNA from oxidative damage. Yu and others (*133*) using an *ex-vivo* test evaluated the ability of hard wheat brans to inhibit the Cu(II) ion induced oxidation of LDL. This report measured LDL oxidation using the thiobarbituric acid reactive substance (TBARS) lipid oxidation method. It

reported results ranging from 1.03 to 1.56 mg TBARS reduction per g bran per 100 μg protein. Another recent study by Liyana-Pathirana and others (*129*) the conjugated dienes method to assess the ability of wheat bran to inhibit LDL oxidation. They reported results of 3315-3795 and 4845-4978 μg LDL protein protected per gram of deffatted wheat bran for soft and hard wheat grains and brans, respectively. Liyana-Pathirana and others also (*118, 129*) reported the dose-dependent ability of wheat extracts to inhibit the hydroxyl radical induced clevage of supercoiled DNA. They found wheat pearling fractions to be more effective than pearled wheat. Krings and others (*146*) demonstrated the ability of roasted wheat germ extract to inhibit damage to DNA induce by a Fe(II)/H_2O_2 ROS generating system.

Of the classes of antioxidative compounds present in wheat, phenolic acids are thought to be one of the primary groups responsible for the ROS scavenging and metal ion chelating properties of wheat observed in-vitro and ex-vivo studies. They may also be the primary bioactive factor responsible for the health promoting properties of whole wheat observed in epidemiological studies. Phenolic acids are known to be concentrated primarily in the bran milling fraction of wheat. This knowledge along with the recent promotion of whole-grain consumption by US Dietary Guidelines has generated renewed research interest in the development wheat based food products which include the bran fraction.

1.8 Functional and Nutraceutical Food Development and Antioxidants

The link between food and health has long been recognized. It was not until the early 20th century, however, that vitamins were identified, and scientific evidence began linked dietary components to chronic disease. This led to fortification of food products to prevent deficiencies in the 1920's, and promotion of diets with reduced levels nutrients such as fat, cholesterol, sodium in the 1970's (*147, 148*). The most recent development in the evolution of food, nutrition, and health has produced a new multidisciplinary research area referred to as "nutraceutical and functional food development". This field evolved from substantial scientific evidence linking diet to health promotion and disease, increasing healthcare costs associated with chronic diseases of aging, the 'self-care' movement with increasing consumer interest in proactively managing health and wellness through diet in addition to traditional medicine, technological advances in biotechnology and genomics, and regulatory approval of health claims on food products (*149*). The term nutraceutical was coined by Stephen DeFelice in the 1990's combining the terms nutrition and pharmaceutical, in reference to food or food substances providing health or disease preventative benefits beyond basic nutritive value (*150*). Similar to nutraceuticals, functional food research goes beyond basic nutrition, striving through sound scientific research to develop food-based bioactives to deliver health promoting properties with proven safety and efficacy (*151*). Dietary supplements are dietary substances which provide health benefits beyond their basic nutritive value delivered in the form of tablets or capsules and not "conventional food" (*152*). While there is no clear distinction

between dietary supplements and functional/nutraceutical foods from a health benefit standpoint, there are clear differences in how the two are regulated in the U.S. including the types of health claims they can contain (*151, 152*). Other terms often used synonymously with nutraceuticals, functional foods, and dietary supplements include pharmafoods, phytochemicals, zoochemicals, and designer foods (*153*).

The potential of nutraceutical and functional foods from an economic and public health perspective has generated tremendous research growth in the last decade. There is a need, however, during their development processes for sound scientific evidence to establish their safety and efficacy to ensure their success (*148, 151, 154, 155*). Once food bioactives have been identified, the evaluation of efficacy should include establishing their bioavailability, pharmacokinetic behavior, stability, necessary intake levels, and mechanisms of action (*81, 151*). Once these are established, data supporting the role of the bioactive compound in providing the proposed health benefits must be established preferably using the "gold-standard" replicated, placebo-controlled, randomized, clinical intervention studies with humans (*148*). Depending on the degree of scientific evidence from these and other studies, several types of health-related claims can be approved by the U.S. Food and Drug Administration (FDA). While recent regulatory changes have made possible "authoritative statements" or "qualified health claims" based on lower levels of scientific consensus to support claims, health claims are still the most scientifically sound way to convey the relationship between bioactive and health condition to consumers, and require "significant scientific agreement" for approval from FDA (*151*). Lastly, the safety of food bioactives should be established for efficacious

levels, and final verifications of efficacy and safety should be included for the bioactive in a final food vehicle (*151*).

Combined use of sound science and cooperation among industry, multidisciplinary academic research groups, and government regulatory agencies is necessary to ensure the success and safety of nutraceutical and functional foods.

1.9 Bioavailability of Phenolic Acids in Wheat

Recent research has identified wheat phenolic acids as a potential bioactive factor responsible for the health promoting properties of whole-wheat. A next step in the development of wheat phenolics into functional and nutraceutical foods requires an understanding of their bioavailability. Bioavailability is a term borrowed from pharmacology dealing with the portion of an oral dose that reaches systemic circulation (*156*). In reference to food-based bioactives, bioavailability includes: 1) availability of bioactive for absorption in the gastrointestinal (GI) system also referred to as 'bioaccessibilty', 2) physical absorption of bioactive through small or large intestinal epithelium, 3) metabolism before during or after metabolism by phase I and II enzymes, 4) tissue distribution, and 5) bioactivity (*156, 157*). Numerous studies involving pure phenolic acids and phenolics in wheat have investigated aspects of their bioavailability with *in-vitro*, animal, and human studies.

Several studies have evaluated the absorption characteristics of both soluble free forms of several phenolic acids present in wheat, as well as soluble conjugated forms. Three animal studies have shown efficient absorption of soluble free forms of ferulic, *p*-coumaric, and caffeic acids in rats with up to 50% recovery in urine of

conjugated forms of these phenolic acids (*158-160*). Two human studies evaluated the absorption of ferulic acid after ingesting soluble free ferulic acid in different food matrices by measuring urinary excretion (*161, 162*). These human studies found relatively high absorption efficiency with up to 25% of ferulic acid absorbed and peak urinary excretion between 7 and 9 hours. In addition, significant amounts of the ferulic acid absorbed were found to be glucuronidated derivatives of ferulic acid indicating phase II metabolism (*161, 162*). Results from a rat study by Zhao and others (*163*) have also shown that a soluble conjugated form of ferulic acid (5-*O*-feruloyl-L-arabinofuranose) was absorbed in small intestine of rats, indicating that soluble conjugated phenolic acids may be absorbable in humans (*163*). Lastly, *in-vitro* studies by Konishi and others using intestinal Caco-2 cells to determine absorption mechanisms for phenolic acids have shown transepithelial transport of pure ferulic and *p*-coumaric acids across monolayers by monocarboxylic acid transporters (*164, 165*). Together, these studies have shown that soluble free forms and potentially soluble conjugated forms of phenolic acids present in wheat can be absorbed in humans. It is of interest, however, to understand how the wheat matrix may influence the bioavailability of wheat phenolics.

It is known that the majority of phenolic acids present in wheat are in an insoluble bound form, esterified to cell wall materials. A recent human study by Kern and others (*166*) which evaluated the recovery of phenolic acids in plasma and urine after wheat bran consumption found only 3% of the total phenolics (soluble free, soluble conjugated, and insoluble bound) were absorbed after 24 hours. This 3% corresponded approximately to the soluble free phenolic contents of the wheat bran

consumed. This study also concluded that the site of absorption was primarily the small intestine (SI), with maximum absorption 1 to 3 hours after ingestion.

While it is well accepted that while soluble free and conjugated wheat phenolics may be absorbed in the human SI, the majority of wheat phenolics are in an insoluble bound form impairing their absorption and making them not bioavailable (160, 166-168).

1.10 Treatments to Improve the Bioavailability of Wheat Phenolic Acids

One possible strategy to improve the bioavailabilty of wheat phenolics is to release the insoluble bound phenolic acids from their matrix prior to consumption using post-harvest treatments. Several studies have investigated the polysaccharide composition of wheat bran cell walls, and the chemical linkages between these polysaccharides and phenolic acids. Studies by Rhodes and others (*169*) and a review by Mathew (*170*) have reported the polysaccharide composition of wheat bran aleurone and pericarp cell walls to include mostly arabinoxylans with some β-glucans and cellulose (*169, 170*). Phenolic acids are primarily esterified to the C-5 hydroxyl group of α-L-arabinofuranosyl substituents which are linked to C-2 or C-3 on the xylopyranosyl backbone. (*170, 171*). Potential post-harvest strategies to release insoluble bound phenolic acids could include treatment with enzymes targeting these specific linkages in wheat bran, or use of fermentation systems which produce these enzymes.

Enzymatic hydrolysis of bound phenolic acids from cell wall materials

including wheat bran has been previously investigated to produce a natural source of ferulic acid for flavor or pharmaceutical application (*170*). This enzymatic approach has been shown to be effective using xylanases, β-gluconases, and cellulases to break up wheat bran cell wall material combined with enzymes specifically to hydrolyze the ester linked phenolic acids such as cinnamoyl or feruloyl esterases (*170-178*). Commercial enzyme preparations containing mixtures of these enzymes have been reported including Ultraflo-L, Viscozyme-L, Celluclast 1.5L, Termamyl, and Lallzyme and shown to effectively release phenolic acids (*172, 177, 179*). One study by Faulds and others (*177*) reported release of 90% of insoluble bound ferulic acid as a result of treatment using the Ultraflo-L enzyme preparation. Together these studies demonstrate that use of enzymatic hydrolysis is a possible approach for releasing bound wheat phenolic acids. All studies to date, however, have conducted enzyme reactions in aqueous systems. These systems which may prove impractical for commercial scale post-harvest treatments for food ingredients because products must be separated from the aqueous reaction phase.

In contrast to aqueous reaction reactions, solid-state reactions take place on a solid support in the absence of free liquid (*180*). Solid-state reaction systems are practical for food ingredient production because they require no expensive equipment, are environmentally friendly, and require little post-reaction processing to recover products because there is no need to remove free liquid at the end of the process to isolate or use products. Solid-state enzymatic procedures have been developed and used to improve the health promoting properties of several botanical materials. Yu and others (*182*) reported use of a solid-state xylanase system to

improve the physiochemical and functional properties of psyllium including significant decreases in water-holding and gelling properties. Lopez and others (*183*) reported the use of a solid-state α-amylase and glucoamylase system to hydrolyze chestnut starch for simultaneous fermentation to produce ethanol.

Besides the use of pure enzymes, solid-state treatments can also be utilized in which microorganisms produce the desired hydrolytic enzymes. Solid-state fermentation is widely used commercially to produce foods such as tempeh, miso, and soy sauce (for the koji step) (*180, 181*). The biochemical nature of fermentation is enzyme-catalyzed reactions. McCue and others (*181*) reported the use of solid-state fermentation with *Rhizopus oligosporus* to produce glycosidases capable of hydrolyzing phenolic acid in soybean powder, increasing its soluble free phenolic acid contents by as much as 255%. A study by Zheng and Shetty (*148*) reported the use of β-glucosidase produced by *Lentinus edodes* to hydrolyze soluble conjugated phenolic acids present in cranberry pomace. Topakas and others (*185*) reported the use of *Sporotrichum thermophile* to produce cinnamoyl esterases and xylanases in a solid-state fermentation system to hydrolyze corn cob phenolic acids. Lastly, Correia and others (*186*) improved the extractable phenolic acid and antioxidant properties of pineapple residue using *Rhizopus oligosporus* to produce glycosidases. These studies demonstrate the potential of using solid-state fermentation treatments to hydrolyze phenolics from botanical food matrices.

Of the reported potential microorganisms for solid-state fermentation, *Saccharomyces cerevisiae* is a particularly attractive option for food ingredient production given its GRAS (generally regarded as safe) status for food products

(*187*). Strains of *S. cerevisiae* have been shown to produce enzymes with a variety of hydrolytic activities. Hernandez and others (*188*) reported the presence of β-glucosidase activity in wild *S. cerevisiae* strains used in wine productions. Lomolinoi and others (*189*) reported the presence of carboxylesterase activity in another strain of *S. cerevisiae* used in wine fermentation. Lastly, Coghe and others in 2004 reported the presence of feruloyl esterase activity in *S. cerevisiae* preparations used in beer fermentation, capable of releasing ferulic acid during beer processing (*190*).

While several studies have examined the use of solid-state fermentation or pure enzyme systems to release phenolic acids from botanical materials, none to date have explored these opportunities for the release of wheat bran phenolic acids. Use of either pure enzyme or yeast fermentation solid-state treatments may therefore represent a novel and commercially practical approach for improving the antioxidant properties of wheat bran.

1.11 Effects of Processing on Antioxidants in Food Systems

Beyond the development of post-harvest treatments to release insoluble bound phenolic acids from wheat, it is also of interest to evaluate the effects that processing may have on wheat antioxidants in wheat-based food systems. Understanding these relationships could help food producers optimize processing conditions to maximize the antioxidant properties of wheat based food systems, as well as their bioavailability. Food processing induces complex physical and chemical changes in food products as a result of added thermal, mechanical, or radiative energy, and changes in moisture content, oxygen content, pH, water activity, enzyme activities,

and trace metal ion concentrations. These changes have the potential to alter antioxidant physicochemical properties in food systems such as their antioxidative scavenging or chelating properties, and their bioavailability in humans.

Two recent reviews have summarized the potential effects that processing may exert on the antioxidant properties of food systems. Nicoli and others (*191*) and Kalt (*192*) identified several mechanisms through which food antioxidant properties can be altered. Decreases in antioxidant properties could result from decreased concentrations of antioxidants from thermal or oxidative degradation, or leaching of these compounds (*191, 192*). In addition, isomerization of compounds to lower antioxidant capacity isomers, formation of new pro-oxidant compounds, or consumption of antioxidant compounds as reactants in chemical reactions such as the Maillard reaction can decrease the antioxidant properties of food systems (*191, 192*). Increases, on the other hand, could be a result of chemical changes which increase the antioxidant capacity of compounds including isomerization, release of compounds from food matrices, and formation of new antioxidant compounds such as Maillard reaction products (*191, 192*). These reviews concluded that combinations of these mechanisms can alter the antioxidant properties in food systems dependent on processing conditions, formulation, antioxidant compounds present, and food matrix.

Similar to other bioactive compounds such as vitamins, food processing has the potential to alter the bioavailability of antioxidants in food systems. Processing can increase bioavailability of compounds through chemical or enzymatic reactions that hydrolyze or release them from food matrix materials which inhibit their absorption. Processing can also physically incorporate fat soluble compounds into

the lipid phase of a food system which improves their absorption (*156, 193*). Reducing particle size through processing has the potential to improve bioavailability by improving digestive extraction and absorption of compounds (*193*). Isomerization of compounds as a result of processing can also alter bioavailability depending on which isomer is more readily absorbed (*156*). Lastly, processing which increases the interactions of the compound with food matrix materials can also decrease bioavailability by causing absorption interference, for example by inhibiting micelle formation for lipid soluble compounds (*193*).

1.12 Effects of Processing on Antioxidants in Non-Wheat Food Systems

The effects of processing on the antioxidant properties and bioavailability of carotenoids, tocopherols, phenols, and Maillard reaction products in wheat-based food systems including have been investigated in several studies. These studies have evaluated these effects for both pure compound model systems and in numerous non-wheat food matrices.

Carotenoids are a class of compounds which have in common a long polyene chain composed of eight isoprenoid units. The different derivatives of this basic structure result from reactions on this polyene chain including cyclization, hydrogenation, dehydrogenation, or insertion of oxygen (*193*). Processing is thought to affect the provitamin A activity of carotenoids through enzymatic or non-enzymatic oxidation reactions (*193*). Lipoxygenase for example, can indirectly oxidize carotenoids by generating free radicals which oxidize carotenoids (*193*). Maceration or mixing of food products which exposes carotenoids to these enzymes

can therefore degrade carotenoids, and mild blanching can inactivate these enzymes and prevent oxidative destruction (*193*). While oxidation is thought to decrease the provitamin A activity of carotenoids, its effects on the antioxidant properties of carotenoids has not been well studied.

Carotenoids exist predominately in nature in an all-*trans* configuration, but can be isomerized to *cis*-configurations upon addition of light, heat, or oxygen (*193*). Isomerization of carotenoids may alter both antioxidant properties and bioavailability depending on the specific carotenoid. Studying the intestinal uptake of β-carotene in gerbils, Deming and others found all-*trans*-β-carotene to be more bioavailable than 9-*cis* or 13-*cis*-β-carotene (*194*). Bohm and others (*195*) studied the $ABTS^{\bullet+}$ scavenging capacity of different geometrical isomers of α-carotene, β-carotene, lycopene, and zeaxanthin and found that isomerization does significantly influence free radical scavenging capacity against this free radical.

Numerous studies have evaluated the influence of processing on carotenoid composition of food systems. Chen and others (*196*) evaluated the effects of high temperature short time (HTST) processing at 105-120ºC on carrot carotenoids, and found thermal processing to significantly isomerize β-carotene leading to formation of 13-, and 15-cis-isomers of β-carotene and lutein. Marx and others (*197*) and Mayer-Miebach and others (*198*) found similar results for processed carrot products, with significant isomerization of β-carotene to *cis*-isomers. Updike and others (*199*) found thermal canning processing to increase the *cis*-isomers of lutein and zeaxanthin by 17-20%. Dewanto and others (*200*) found the thermal processing of tomatoes to

increase the *trans*-lycopene contents, while increasing the total oxyradical scavenging capacity and bioaccessible lycopene contents. This increase in antioxidant capacity was attributed in part to the release of lycopene from the food matrix components which improve their extractability and therefore bioaccessibility. Sanchez-Moreno and others (*201*) found high-pressure processing to increase the carotenoid contents tomato puree. Other studies on bioavailabilty, have found mild thermal processing to improve carotenoid bioavailabilty by denaturing protein-carotenoid complexes which otherwise inhibit their absorption (*202*).

Tocopherols include methyl substituted derivatives of a saturated isoprenoid side chain attached to a chroman-6-ol ring. Tocotrienols have identical structures but with three *trans* double bonds in the side chain (*193*). Tocopherols and tocotrienols (tocols) are susceptible to light, heat, and oxidative degradation during processing. Similar to carotenoids, tocols are susceptible to oxidative damage. Oxidation of tocols can result in numerous products including quinones, dimmer, trimers, and epoxides (Bramley et al 2000). Similar to carotenoids, lipoxygenase enzymes present in many plant tissues can indirectly degrade tocols through lipid peroxidative products during maceration or mixing processes (*193*). Non-enzymatic oxidation of tocols is thought to be accelerated by heat, basic conditions, trace metal ions, and exposure to lipid peroxidation products (*203*).

The effects of processing on the tocols composition of non-wheat food systems have been reviewed extensively. Reviews by Bauernfeind (*204*), Bramley (*203*), Eitenmiller and Lee (*205*), and Ball (*193*) found most heat treatments including dehydration, baking, microwaving, canning and frying as well as irradiation to

significantly decrease tocopherols by as much as one-third. Other processes such as extrusion, flaking, puffing, and shredding are known to be even more degradative, reducing tocols by 40-83%.

The effects of processing on polyphenols and phenolic acids contents and compositions in food systems have been evaluated, although less thoroughly than antioxidative vitamins such as carotenoids and tocols. Larrauri and others (*206*) showed significant decreases in polyphenols, tannins, and antioxidant capacity of red grape pomace peels subjected to temperatures of 100ºC and above. A study evaluating thermal processing in sweet corn found decreases in vitamin C contents from increasing temperature (100-150 ºC) or time (0-50 min), while soluble free and conjugated phenolic acid contents increased as a result of released bound phenolics (*207*). Gahler and others (*208*) found thermal processing to increase the total phenolic contents and ABTS cation radical scavenging capacity of tomato products. A recent study by Jiratana and others (*209*) found no change in total antioxidant activity in beets as a result of thermal processing, while thermal treatments of green beans showed decreases in phenolic acids contents. Choi and others (*210*) found increasing heat treatments from 100 to 121 ºC to increase the free polyphenolic contents of Shiitake mushrooms 1.9-fold, while also increasing cation ABTS and DPPH radical scavenging capacities by 2- to 2.2-fold. Jeong and others (*211*) found increasing heat treatment from 50-150 ºC to significantly increase the antioxidant properties of citrus peels including radical scavenging properties and formation of low molecular weight phenolic compounds. Dupas and others (*212*) found addition of milk to coffee to decrease up to 40% the available chlorogenic acid through its

binding to milk proteins. Bryngelsson and others (*213*) found autoclaving of oat grains to significantly increase extractable phenolic acid contents, while drum drying resulted in large decreases in the same phenolic acids. Lastly, Sensoy and others (*214*) found roasting of buckwheat at 200ºC for 10 minutes to not significantly change its extractable total phenolic contents while decreasing its extractable DPPH radical scavenging capacity. In summary, the effects of processing on the phenolic contents of food systems are highly dependent on food matrix and processing step utilized. In addition, few studies have investigated the effects of processing on the bioavailability of phenolic compounds.

The Maillard reaction is a complex series of non-enzymatic reactions involving a reducing sugar and an amino acid which can take place during the processing in numerous food systems. Maillard reaction products (MRPs) are numerous and include some which are thought to have antioxidant properties such as high molecular weight melanoidines (*215, 216*). Other MRPs may have pro-oxidant properties (*215, 216*). Numerous factors influenced by food processing such as temperature and water activity can influence the products formed in the Maillard reaction (*217*). The majority of studies to date have used model systems to study the formation of antioxidant MRPs, while some recent studies have used complex food systems.

Yilmaz and Toledo (*218*) used a model reaction system with histidine and glucose and found MRPs with significant peroxyl radical scavenging to be formed at an optimal temperature of 120ºC for times from 10-30 min. Benjakul and others (*217*) utilized a porcine plasma protein model system with different reducing sugars

to study the formation of antioxidant compounds at 100ºC. This study found MRPs derived from galactose to have significantly higher DPPH radical scavenging activity compared to those derived from fructose or glucose. Hayase and others (*219*) reported the formation of a MRP from a D-xylose and glycine model system with antioxidant properties comparable to that of BHA and BHT. Fuster and others (*220*) reported increases in coffee antioxidant activity as a result of roasting even though polyphenol contents decreased, suggesting a role of MRPs.

Several studies have also suggested the formation of pro-oxidant compounds as a result of the Maillard reaction in food systems. Duh and others (*221*) reported the decrease in barley antioxidant properties as a result of roasting, even though roasting increased MRPs. A report by Anese and others (*222*) found short heat treatments of tomato juice to its decrease antioxidant properties possibly from the formation of early MRPs, while prolonged heating overall increased the antioxidant activity of the same samples. This has been supported by several studies indicating that low temperature or short-time heat treatments may lead to higher levels of pro-oxidant radicals formed in the early stages of the Maillard reaction (*191*).

1.13 Effects of Processing on Antioxidants in Wheat Food Systems

While it has been hypothesized that processing of whole-grain food products may increase or decrease antioxidant properties and bioavailability, few studies to date have evaluated these effects (223). Given the predominate use of wheat in leavened bakery type products, the potential processing steps of interest involved for wheat based food products can include: size reduction of wheat milling fractions,

mixing of dry ingredients with water, kneading, fermentative proofing, and baking. Together these processing steps have the potential to significantly alter wheat antioxidant properties and bioavailability as a result of thermal, mechanical, chemical, physical, and enzymatic changes.

Several studies from the 1990's examined the influence of processing on wheat-based food system antioxidative vitamin contents. These studies, however, were not specifically interested in antioxidant properties changes but rather the effects on vitamin contents. Rogers and others (*224*) evaluated the stability of β-carotene during baking and pre-baking processing steps for yellow cake, sugar cookie, and bagels. This study found pre-baking processing steps such as mixing, rolling, and proofing to cause no significant decreases in β-carotene. This study also found baking to cause significant reductions in the all-*trans* β-carotene isomer contents ranging from 74-85%. Ranhotra and others (*225*) found β-carotene fortified bread and cracker baking to decrease total carotenoids from 4-23% with up to 20% of remaining carotenoids isomerized to 13- or 9-*cis* isomers. This study also found pre-baking steps for these products including mixing and proofing to cause minimal decreases in total carotenoids. Evaluating the effects of semolina pasta dough mixing on antioxidant contents, one study found this processing step to significantly decrease the contents of carotenoids (*226*). A recent study by Leenhardt and others (*227*) found the most significant decreases (66%) in total carotenoid contents during bread making to occur during kneading with a high correlation of these losses to the lipoxygenase activity of wheat variety. This same study found less than 10% of losses in total carotenoids as a result of dough fermentation at 30 ºC, and losses

during baking of 36-45%. Together, these studies indicate that food processing in wheat-based food systems can alter its β-carotene contents and induce isomerization. The magnitude of these changes, however, are dependent on the food matrix and processing steps utilized.

Food processing induced changes in the antioxidative vitamin E contents of wheat food products have been investigated. Hakansson and Jagerstad (*228*) evaluated the effects of mixing and drum drying on the vitamin E retention of wheat flours. Their study found mixing of water with flour to induce significant losses of vitamin E likely from lipoxygenase activity, and continued processing with drum drying to degrade 90% of vitamin E present. Hakansson and Jagerstad (*229*) evaluated the effect of bread making steps on vitamin E losses and found fermentation to have no effect on vitamin E levels, while sour dough fermentation and dough mixing resulted in 20-60% decreases in vitamin E contents. Ranhorta and others (*230*) found vitamin E fortified bread to retain 67% of the original vitamin E contents after all processing steps including mixing, kneading, and baking. A recent study by Leenhardt and others found significant decreases in the tocopherol contents during bread making with 10-12% losses attributed to kneading, and 15-20% losses attributed to baking (*227*). Further evaluating these changes, the study attributed the losses of vitamin E to direct oxidation and thermal destruction of vitamin E during processing (*227*). Together, these studies indicate that food processing in wheat-based food systems can alter its vitamin E contents. The magnitudes of these changes, however, are dependent on the food matrix and processing steps utilized.

The effects of food processing on phenolic acids in grain-based foods have

been investigated.. A study by Hansen and others (*231*) evaluating changes in phenolic acid contents of whole-grain sourdough rye bread during processing and found dough mixing, proofing, and baking processing steps to all decrease total ester-bound phenolic contents with a total decrease of 7% observed. This same study found an increase from 3 to 12 μg/g in soluble free ferulic acid contents of rye bread after mixing and fermentative proofing at 20ºC for 16 hr. Another study with whole-grain sourdough rye bread found dough fermentation to increase its soluble free phenolic acid contents (*232*). Evaluating the effects of semolina pasta dough mixing on antioxidant contents, one study found this processing step to significantly decrease the contents of ester-bound ferulic acid (*223*). A study by Gelinas and McKinnon (*127*) found the bread-baking process to slightly increase the total phenolic contents of refined wheat bread. In summary, few studies have evaluated processing induced changes in whole-grain food products phenolic acids, and no studies have been done specifically with whole-wheat food products.

The formation of Maillard reaction products in wheat-based food systems have been implicated in the changes in antioxidant properties observed in at least two studies. Bressa and others (*233*) reported that enhanced browning reactions in baked cookies from added sugar and amino acids increased their oxidative stability. Another study by Lindenmeier and others (*234*) found increasing concentrations of the antioxidant Maillard reaction product 2,4-dihydroxy-2,5-dimethyl-1-(5-acetamino-5-methoxycarbonyl-pentyl)-3-oxo-2H-pyrrol (pronyl-L-lysine) in bread crust as a result of increasing thermal treatments from 220-260ºC and 70 to 210 min. This study also found the addition of lysine-rich casein or glucose to bread formulations to increase

production of the antioxidant compound (pronyl-L-lysine) by more than 200%. These studies support the role that the Maillard reaction may play in altering the antioxidant properties and contents of wheat-based food systems.

1.14 In-vitro Methods to Assess the Antioxidant Properties of Wheat

Two commonly used types of assays for evaluating the antioxidant properties of wheat include compositional analysis for specific classes of antioxidant compounds, and *in-vitro* antioxidant capacity assays. *In-vitro* methods are used to help access the potential effectiveness of antioxidants *in-vivo*. These *in-vitro* methods have several advantages for development of nutraceutical and functional foods compared to *in-vivo* tests. *In-vitro* methods cost significantly less than *in-vivo* tests and can more quickly screen the antioxidant capacity of large numbers. In addition, with greater sensitivity and less interference from confounding variables, *in-vitro* methods can more easily elucidate the effects experimental variables in nutraceutical development. Given the numerous mechanisms through which antioxidants can function in biological systems, it has become necessary to develop numerous methods to access the diverse properties of dietary antioxidants.

1.14.1 Phenolic Acid Composition Analysis

The phenolic acids composition of wheat including free soluble, conjugated soluble, and insoluble phenolic acids can be determined using a protocol described by Moore and others (*93*). The free soluble, conjugated soluble, and insoluble phenolic acids are extracted following a combined solvent and pH extraction and fractionation, and alkaline catalyzed release of bound phenolic acids from the solid grain matrix.

Phenolic acid composition for extracts dissolved in methanol is analyzed by HPLC using a C18 column. Phenolic acids are separated using a linear gradient elution program with mixtures of acetic acid and H_2O. Identification of phenolic acids is accomplished by comparing the retention time of peaks in the MeOH solution to that of the standard compounds using HPLC with photodiode array detector. Quantification of individual phenolic acid is conducted using total area under each peak with external standards.

1.14.2 Carotenoid and Tocopherol Composition Analysis

Carotenoids and tocopherols can be extracted and analyzed following a previously reported procedure using high performance liquid chromatography-diode-array-detector-electrospray ionization tandem mass spectrometry (HPLC-DAD-ESI-MSMS) (*235*). HPLC analysis is performed using a TSQ Quantum tandem mass spectrometry equipped with an ESI interface and Agilent 1100 HPLC system. HPLC separation is accomplished according to a previously described protocol with modifications (*235*). A Zorbax RX-SIL column (2.1 mm i.d. × 150 mm, 5 μm particle size; Agilent Technologies, Palo Alto, CA, USA) is used at room temperature. The carotenoids are eluted using a mobile phase of hexane as solvent A and 1% i-PrOH in EtOAc as solvent B. The gradient procedure is as follows: 1) the gradient is linear from 1% to 10% of solvent B and the flow rate is increased from 0.50 to 1.00 mL/min in the first 5 min, and 2) 10% of solvent B is increased to 40% and flow rate was kept same at 1.00 mL/min from 5 to 25 min. Identification of α-tocopherol and the four carotenoids is accomplished by comparing the HPLC

retention time and selected reactant monitoring (SRM) analysis of the sample peaks with that of the authorized pure commercial α-tocopherol and carotenoid compounds. The quantification for α-tocopherol and each carotenoid compound is conducted using the total ion counts with an external standard and mass spectrometer detector.

1.14.3 Total Phenolic Contents (TPC) Assay

The total phenolic contents (TPC) assay is a simple spectrophotometric method to estimate the total phenolic contents of samples by measuring its reducing capacity with the Folin-Ciocalteu (FC) reagent. The method measures the ability of samples under basic conditions to reduce the yellow FC reagent causing its color to change to a dark blue. Under basic conditions, phenolic groups are deprotonated leading to a phenolate anion with reducing potential. The chemical nature of the FC reagent is not entirely understood but is thought to be a phosphomolybdate-phosphotungstate complex (*236*). It is believed that molybdenum in this complex, Mo(VI) has the characteristic yellow color, which upon reduction to Mo(V) by phenolate anions becomes blue (*236*). This method was developed by Singleton and others (*237*) and quantifies "total phenolics" or reducing capacity in reference to that of a standard phenolic acid, gallic acid, with results expressed as mg gallic acid equivalents per g sample. The major advantage of this assay is operational simplicity. The major disadvantages of the TPC assay include a lack of specificity in complex botanical extracts for phenols which the assay was designed to measure. This assay also lacks of a buffered reaction system causing changes in reaction pH from samples which may affect reducing capacity measurements.

1.14.4 Total Flavonoid Content Assay

The total Flavonoid contents of botanical extracts can be determined with this spectrophotometric method reported by Adom and others (*94*). Flavonoids in this assay react with sodium nitrite and aluminum chloride to form a flavonoid-aluminum complex which absorbs at 510 nm. Results for this assay are expressed relative to catechin as a standard. While this assay is a relatively simple method, little data is available to confirm the specificity of this assay for flavonoids, and potential interfering compounds.

1.14.5 DPPH Radical Scavenging Capacity Assay

The DPPH radical (DDPH$^{\bullet}$) assay is a decolorization assay which measures the capacity of antioxidants to directly react with (scavenge) DPPH radicals by monitoring its absorbance at 517 nm with a spectrophotometer. The DPPH radical is a stable organic nitrogen centered free radical with a dark purple color. When the DPPH radical is reduced to its non-radical form by antioxidants it becomes colorless. The recently developed Relative DPPH Scavenging Capacity (RDSC) version of this method is a simple and adaptable for high-throughput analysis using 96-well plates. It takes into account both kinetic and thermodynamic properties of antioxidants, has broad solvent compatibility allowing analysis of hydrophilic and lipophilic antioxidants, and uses a standard allowing for comparison of results between labs (*132*). The major disadvantage of this assay is the use of a non-physiologically relevant radical, the DPPH radical which has little resemblance to free radicals involved in oxidative processes in biological systems.

1.14.6 ABTS Cation Radical Scavenging Capacity Assay

The ABTS cation radical ($ABTS^{\bullet+}$) assay is a decolorization assay which measures the capacity of antioxidants to directly react with (scavenge) ABTS cation radicals. $ABTS^{\bullet+}$ is a nitrogen centered radical with a characteristic blue-green color which when reduced by antioxidants to its non-radical (ABTS) form becomes colorless. The method quantifies scavenging capacity by measuring the absorbance at a fixed time of the reaction mixture at 734 nm with a spectrophotometer. Results are expressed relative to a standard, trolox. This method, developed by Miller and others (*238*) is operationally simple with quick analysis times, and with broad solvent compatibility allows for analysis of hydrophilic and lipophilic antioxidants. The major disadvantages of this assay are use of fixed time point measurements which do not adequately take into account both kinetic and thermodynamic properties of antioxidants, and use a non-physiologically relevant radical.

1.14.7 Superoxide Anion Radical Scavenging Capacity Assay

The superoxide anion radical ($O_2^{\bullet-}$) scavenging capacity assay was developed to evaluate the ability of hydrophilic antioxidants to scavenge this physiologically relevant radical. Two assays types of assays have been reported for evaluating wheat antioxidant properties against the superoxide anion radical. The first is a spectrophotometric assay which measures the ability of sample extracts to compete with a molecular probe, nitroblue tetrazolium (NBT) and scavenge $O_2^{\bullet-}$ generated by an enzymatic hypoxanthine-xanthine oxidase (HPX-XOD) system. NBT has a yellow color which upon reduction by $O_2^{\bullet-}$ forms formazan with a blue color

measurable at 560 nm with a spectrophotometer. This assay quantifies $O_2^{\bullet-}$ scavenging capacity as percent $O_2^{\bullet-}$ remaining. This method was developed by Gaulejac and others (*239*) and has the advantage versus other methods of evaluating scavenging capacity against a physiologically relevant ROS. The major disadvantages of this assay include potential interferences between antioxidants and the enzymatic ROS generating system, potential generation of interfering H_2O_2 by this system, lack of standard making results difficult to compare between labs, and use of a fixed time point measurement which does not adequately account for both kinetic and thermodynamic properties of antioxidants.

The second assay reported for $O_2^{\bullet-}$ scavenging capacity is an electron spin resonance (ESR) assay using spin-trapping techniques. This assay described for wheat antioxidant evaluation by Zhou and others (*240*) utilizes the xanthine/xanthine oxidase enzymatic system to generate $O_2^{\bullet-}$. It detects the concentration of $O_2^{\bullet-}$ after addition of antioxidant extract using 5-tert-butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO) as a trapping agent. Results for this assay can be expressed as percent $O_2^{\bullet-}$ or relative to a standard such as superoxide dismutase (SOD). The major advantage of this version of the $O_2^{\bullet-}$ scavenging capacity assay is more specific detection of $O_2^{\bullet-}$ using an ESR spin-trapping assay. The major disadvantages include the cost and extensive operator skill required for ESR, and similar to the spectrophotometric method, the possibility of interference by inhibiting the enzymatic $O_2^{\bullet-}$ generating system.

1.14.8 Oxygen Radical Absorbing Capacity (ORAC) Assay

The oxygen radical absorbing capacity (ORAC) assay was developed to measure the hydrophilic chain-breaking antioxidant capacity of sample extracts against the peroxyl radical through a hydrogen atom transfer (HAT) mechanism (*241*). This assay uses competitive kinetics to monitor the ability of sample extracts to compete with a molecular probe, fluorescein (FL), and scavenge peroxyl radicals generated by 2,2-azobis(2-amidinopropane dihydrochloride (APPH), an azo compound. Reactions of peroxyl radicals with FL yield a non-fluorescent product which can be monitored by measuring the loss of fluorescence of FL with a fluorometer. An adaptation of this assay for evaluation of lipophilic antioxidants utilizes randomly methylated β-cyclodextrin (RMCD) as a solubility enhancer (*242*). Results for ORAC are determined using area under the kinetic curve calculations for samples, standards, and a blank. Trolox is used as the standard with results expressed as μmol TE/g. This method was originally developed by Cao and others (*251*) with recent improvements by Huang and others including high-throughput adaptation for 96-wells plates (*241, 243*). The major advantages of this method include the evaluation of scavenging capacity against a physiologically relevant ROS, and use of area under the curve (AUC) measurements which take into account both kinetic and thermodynamic properties of antioxidants. Major disadvantages of the ORAC method include the lack of ESR validation to demonstrate the types of free radicals generated under ORAC conditions, and potential sample solvent interferences which may lead to generation of carbon centered radicals.

1.14.9 Peroxyl Radical Scavenging Capacity (PSC) Assay

The PSC assay, similar to the ORAC assay, was developed to access the peroxyl radical scavenging capacity of both hydrophilic and lipophilic antioxidants (*136*). This method generates peroxyl radicals from the thermal degradation of 2,2'-azobis(amidinopropane), and measures the ability of antioxidants to compete with dichlorofluorescin diacetate, a compound which fluoresces upon oxidation by peroxyl radicals. The lipophilic PSC assay adds randomly methylated β-cyclodextrin (RMCD) to the reaction mixture to enhance the solubility of lipophilic compounds, while the hydrophilic version omits this compound. Results for this method are determined using AUC calculations and expressed with EC_{50} values or relative to standards such as gallic acid or vitamin C for hydrophilic assays or α-tocopherol for lipophilic assays. The major advantage of this assay is its ability to estimate radical scavenging capacity for both hydrophilic and lipophilic antioxidants. Similar to ORAC, however, the lack of ESR validation for the free radical species generated in the PSC reaction system and with organic solvents is the major disadvantage of this assay.

1.14.10 Total Oxyradical Scavenging Capacity (TOSC) Assay

The TOSC assay measures the ability of sample extracts to inhibit the peroxyl radical induced oxidation of α-keto-γ-methiolbutyric acid measured using headspace gas chromatography (GC) (*94, 95*). In this reaction system, peroxy radicals are generated with 2,2'-azobis-amidinopropane (ABAP) which oxidize α-keto-γ-methiolbutyric to form ethylene gas. This method calculates antioxidant capacity

using an EC_{50} calculation with results expressed relative to vitamin C as a standard. The major advantage of this assay is use of a fatty acid-based reaction system to evaluate the ability of samples to inhibit lipid peroxidation. Disadvantages of this assay include potential decreased sensitivity of ethylene gas compared to other lipid oxidation products of higher concentrations, as well as the need for expensive GC equipment with headspace auto sampler to analyze multiple samples.

1.14.11 Hydrogen Peroxide Scavenging Capacity Assay

This assay evaluates the ability of sample extracts to scavenge H_2O_2 by measuring the percent added H_2O_2 remaining after incubation with sample, using a peroxidase system to measure H_2O_2 contents (*137*). While evaluating scavenging activity against a physiologically relevant ROS, this assay does not have an adequate calculation method to compare samples or results between labs, nor does it take into account both the kinetic and thermodynamic properties of antioxidants.

1.14.12 Hydroxyl Radical Scavenging Capacity Assays

The hydroxyl radical is the most reactive ROS in biological systems, making it of interest to understand how wheat components may scavenge this ROS (*47*). The deoxyribose method developed by Aruoma (*244*) has been one of the most popular assays used to evaluate hydroxyl radical scavenging properties of antioxidant compounds. This method uses the classic Fenton reaction with Fe^{2+} (reduced from Fe^{3+} by ascorbic acid) and H_2O_2 in a buffered system to generate $^{\bullet}OH$ and degrade 2-deoxyribose to malondialdehyde-like products which form a chromagen with thiobarbituric acid detectable at 532nm. This method unfortunately suffers from

several drawbacks including a lack of compatibility with commonly used extraction solvents such as ethanol and acetone, and use of fixed time point measurements which do not take into account kinetic and thermodynamic properties of antioxidants (*47*). Given the limitations of this method, there exists a need for development of an improved hydroxyl radical scavenging capacity assay. Another method developed for hydroxyl radical scavenging measurements uses ESR with 5,5-dimethyl *N*-oxide pyrroline (DMPO) as a spin trapping agent (*92*). This method is capable of more directly detecting hydroxyl radical scavenging compared to spectrophotometric methods, but requires expensive ESR equipment and extensive operator experience, and has not been validated for use of standards to enable comparisons of results.

1.14.13 Iron (II) Chelating Capacity Assay

Ferrous irons in biological systems can react with oxidizing agents such as H_2O_2 to form numerous ROS. The Fe^{2+} chelating assay measures the capacity of test compounds to compete with a synthetic chelator to form more stable coordination complexes with iron(II) under physiological pH. The bidentate chelator used in this assay, 2,2'-bipyridine, coordinates with Fe^{2+} forming a red complex which when displaced by higher affinity chelators causes a decrease in red color measured at 522 nm with a spectrophotometer. This method developed by Yamaguchi and others (*245*) uses ethylenediaminetetraacidic acid (EDTA), the common synthetic chelator which as a hexadentate ligand can form more stable complexes with iron(II) than with 2,2'-bipyridine, is used as a standard in this assay. The major advantage of this assay is its ability to measure a property of antioxidant not evaluated by other assays, chelating

capacity. The major disadvantage of this assay is its lack of ability to evaluate other chelator properties which are necessary to understand a compounds ability to inhibit iron induced ROS generation, such as iron(III) selectivity, rigidity of ligand conformations, availability of coordination sites, affinity towards iron ions, and stoichiometry of the complex (*73, 246*).

1.14.14 Copper (II) Chelating Capacity Assay

Similar to iron, copper is a physiologically relevant metal capable of generating ROS *in-vivo*. The capacity of compounds to chelate copper ions rendering them reactively inert is therefore of great interest. Zhou and others (*240*) reported use of an ESR method to determine the Cu(II) chelating capacity of wheat phenolic acids. This method is a simple system involving a sample extract and Cu(II) ions. Chelating capacity is qualitatively determined by examining shifts in the Cu(II) spectra as a result of coordination with antioxidants at low temperatures (77ºK). The major advantage of this assay is the direct measurement of interaction between antioxidant compounds and copper ions using ESR, but like other ESR methods this method requires expensive equipment and extensive operator experience. In addition, this method unlike other antioxidant capacity assays is not quantitative in nature.

1.14.15 Assays Measuring Inhibition of Lipid Peroxidation

Numerous methods have been developed to examine the ability of wheat extracts to inhibit the oxidation of lipids. Three major differences exist between these methods. First, the composition of the reaction systems differ, and can include lipid based systems or emulsified lipid-aqueous systems. For these lipid systems, different

fatty acid compositions can be utilized including pure fatty acids such as linoleic acid, or natural oils extracted from plants or animals. Secondly, oxidation of lipids can be induced through several means including use of ROS generating systems such as the Fe(III)/ascorbic acid system or azo compounds, or utilize accelerated oxidation conditions such as increases in temperature or oxygen availability. Lastly, the development of lipid oxidation can be evaluated using several analytical methods such as the thiobarbituric acid-reactive substances (TBARS) assay, peroxide value (PV) assay, conjugated dienes (CD) method, β-carotene bleaching method, and measurements of volatile organic acids, all of which measure secondary products of lipid peroxidation.

1.14.15.1 Active Oxygen Method

The Active Oxygen Method (official method Cd 12-57) was developed by AOCS (*247)* to standardize accelerated oxidation conditions which could be utilized to evaluate the stability of lipids. Although AOCS now considers this method to be obsolete, the conditions used in this method are still utilized in some lipid oxidation assays.

1.14.15.2 β-Carotene-Linoleic Acid Method

This assay developed by Miller and others (*248*) evaluates the ability of sample extracts to inhibit the autoxidation of linoleic acid in an aqueous emulsion system by monitoring the competitive bleaching of β-carotene which absorbs in the visible region. β-carotene in this assay is de-colorized by peroxides generated during the oxidation of linoleic acid. While this assay evaluates antioxidant activity in a lipid based system, it has been criticized for lack of reproducibility and lack of

calculation method to quantify scavenging activity (*249*).

1.14.15.3 Oxidative Stability Index Method

The antioxidative capacity of extracts measured as inhibition of lipid oxidation can be evaluated using the oxidative stability index (OSI) assay. The OSI method is a standardized accelerated oxidation assay developed to automate the evaluation of fat and oil oxidative stability using two possible pieces of equipment, the Rancimat (Metrohm Ltd, Herisau, Switzerland) or the Oxidative Stability Instrument (Omnion Inc, Rockland, MA, USA) using a protocol (official method Cd 12b-92) outlined by the American Oil Chemists' Society (*247*). In this method, purified air is passed through oil samples held at an elevated temperature, and stability is determined by measuring the conductivity of secondary products lipid peroxidation (*247*). Protocols specific for analysis of wheat samples have been reported by Yu and others (*133*) and Liyana-Pathirana and others (*129*). Results for this assay are expressed as the time period until maximal change in oxidation rate called "induction time", or using a protection factor calculation which compares the induction time of samples to controls. The major advantage of the OSI assay versus other antioxidant capacity assays is its use of a lipid-based reaction system to evaluate antioxidant capacity. Compared to other assay for evaluating extent or inhibition of oxidation in lipid systems such as peroxide value or TBARS, the OSI method is considerably simpler, but requires use of expensive equipment. Other disadvantages include use of accelerated high temperature and oxygen concentration conditions, lower sensitivity compared to TBARS and peroxide value methods, and lack of standardization to help compare results between labs.

1.14.15.4 Inhibition of Low Density Lipoprotein (LDL) Oxidation Method

The inhibition of LDL oxidation assay measures the capacity of samples to prevent copper(II) induced oxidative damage to LDL. Inhibition of LDL oxidative damage is commonly evaluated by measuring the formation of secondary lipid oxidation products with the thiobarbituric acid-reactive substances (TBARS) assay using 1,1,3,3-tetraethoxypropane as a standard or measuring conjugated dienes (*250*). A protocol for evaluating wheat's ability to inhibit LDL oxidation using either of these markers of lipid oxidation have recently been reported (*129, 133*). The major advantage the LDL oxidation inhibition method is its relevance to *in-vivo* conditions including oxidation of LDL which is known to be involved in the development of atherosclerosis, and use of copper(II), a physiologically relevant inducer of lipid oxidation. Major disadvantages of this method include the number of steps and time involved, and the lack of adaptation for high-throughput analysis.

1.14.15.5 Inhibition DNA Oxidation Method

Several assays have been reported to access the antioxidative ability of wheat extracts to inhibit oxidative damage to DNA. A 3-D method reported by Krings and others (*146*) induces damage to plasmid DNA with a hydroxyl radical generating Fenton reaction, and measures damaged DNA by adding DNA damage repair enzyme systems which incorporate luminescent nucleotides into the DNA plasmid. Another recently reported method measures the ability of extracts to inhibit Fenton reaction induced oxidative DNA strand breakage measured using gel electrophoresis. Both of these assays have the advantage of measuring the ability of extracts to inhibit oxidative damage to physiologically relevant biomolecule, DNA (*118*).

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Chapter 2: Novel Fluorometric Assay for Hydroxyl Radical Scavenging Capacity (HOSC) Estimation

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2.1 Abstract

The hydroxyl radical is one of the most reactive free radicals generated in biological systems. A novel fluorometric method was developed and validated for hydroxyl radical scavenging capacity (HOSC) estimation using fluorescein as the probe. A constant flux of pure hydroxyl radical is generated under physiological pH using a Fenton-like Fe^{3+}/H_2O_2 reaction. The generation of pure hydroxyl radicals under the experimental conditions was evaluated and confirmed using electron spin resonance (ESR) with DMPO spin trapping measurements. The hydroxyl radical scavenging capacity of a selected antioxidant sample is quantified by measuring the area under the fluorescence decay curve with or without the presence of the antioxidant and expressed as trolox equivalents per unit of the antioxidant. The assay may be performed using a plate reader with fluorescent detector for high-throughput measurements. The assay was validated for linearity, precision, accuracy, reproducibility, and its correlation with a popular peroxyl radical scavenging capacity assay using selected pure antioxidant compounds and botanical extracts. This method may provide researchers in the food, nutrition, and medical fields an easy to use protocol to evaluate free radical scavenging capacity of pure antioxidants and natural extracts in vitro against the very reactive hydroxyl radical which could be linked to

numerous degenerative diseases and conditions.

2.2 Introduction

Reactive oxygen species (ROS) and their roles in human pathology have recently been of great interest in the area of health promotion and disease prevention. ROS contribute to oxidative stress which is linked to numerous degenerative conditions including carcinogenesis (*1*), cardiovascular disease (*2*), inflammation (*3*), Alzheimer's disease (*4*), Parkinson's disease (*5*), diabetes (*6*), and aging (*7*). Mechanisms involved in the role of ROS and oxidative stress in disease development may include alteration of important biomolecules causing oxidative modification of proteins (*8*), oxidation of lipids (*9*), stand breaks and modification to nucleic acids (*10*), modulation of gene expression through activation of redox-sensitive transcription factors (*11, 12*), and modulation of inflammatory responses through signal transduction (*3*). Growing evidence suggests that dietary antioxidants may prevent important biological molecules from oxidative damages, and reduce the risk of numerous chronic diseases related to advancing age (*13*). This has created a demand of simple, reliable, high-throughput *in-vitro* method(s) measured under physiologically relevant conditions for the rapid screening and development of potential dietary antioxidants, as suggested by a recent "white paper" review from the First International Congress on Antioxidant Methods sponsored by the American Chemical Society (*14*).

Of the physiologically relevant ROS, hydroxyl radical ($^{\bullet}OH$) is extremely reactive

with almost every type of biomolecules, and is possibly the most reactive chemical species known (*15*). The presence and pathological role of $^{\bullet}OH$ *in-vivo* have been demonstrated and include direct attack of proteins and nucleic acids (*10, 16*). Hydroxyl radicals may serve as an excellent target to investigate dietary antioxidants for their potential to directly react with and quenching free radicals, and protect important biomolecules from radical mediated damages.

One of the most important properties of an ideal $^{\bullet}OH$ scavenging capacity assay is a source of $^{\bullet}OH$ without interference from other ROS. There are five categories of $^{\bullet}OH$ generating systems described by previous researchers based on the reaction used for radical generation including **1)** The classic Fenton reaction including the pH 7.4 buffered ferric iron-EDTA/ascorbic acid/H_2O_2 system used in the "deoxyribose assay" (*17*), and several alternatives (*18-20*); **2)** The superoxide-driven Fenton reaction known also as the Haber Wilstatter or the Haber Weiss reactions (*21-24*) using the hypoxanthine/xanthine oxidase system to generate $^{\bullet}OH$, and its alternative proposed by Yang and Guo (*25*); **3)** Use of "Fenton-like" reagents to produce $^{\bullet}OH$ including the Co(II)/H_2O_2 (*26*) and the Cu(II)/H_2O_2 systems (*27, 28*); **4)** Pulse radiolysis of water to generate $^{\bullet}OH$ (*15*); and **5)** "Photo-Fenton" systems using photosensitizers to create $^{\bullet}OH$ (*29*). The radiolysis and photosensitization systems do not measure the $^{\bullet}OH$ scavenging capacity under physiologically relevant conditions. The hypoxanthine/xanthine oxidase system generates superoxide ($O_2^{\bullet-}$) in addition to $^{\bullet}OH$, and may have interference from enzyme inhibitors which result in

overestimation of •OH scavenging capacity.

The "deoxyribose method" is one of the most commonly used methods for •OH scavenging capacity estimation in the aqueous phase (*30-33*). This method uses the classic Fenton reaction to generate •OH and degrade 2-deoxyribose to "malondialdehyde-like" products which form a chromagen with thiobarbituric acid detectable at 532 nm. However, our preliminary study observed two issues with this method. First, a drop in pH from 7.4 to 6.0 was found during the initial reaction, and attempting to increase the system buffering capacity to maintain pH 7.4 resulted in no detectable chromagen. Additional experiments using fluorescein (FL) as the probe instead of deoxyribose explained these observations, showing that FL was rapidly degraded at pH 6.0, whereas almost no FL was degraded while at pH 7.4 (**Figure 2.1**), suggesting that "deoxyribose method" may not generate •OH under the physiological pH. This finding was supported by observations of other researchers (*25, 26, 34-36*). The second issue with this assay was an extraction solvent interference. Upon running the assay with 50% acetone solution of antioxidants, no significant difference was found between sample and blank measurements. UV spectrum analyses showed that the chromagen absorption spectrum was altered with the peak at 532 nm significantly decreased (**Figure 2.2**) in the presence of acetone. This could possibly be due to the competition of the carbonyl group in acetone molecules with the "malondialdehyde-like" compounds for the reaction with thiobarbituric acid, thereby forming a different chromagen molecule with altered absorption spectra.

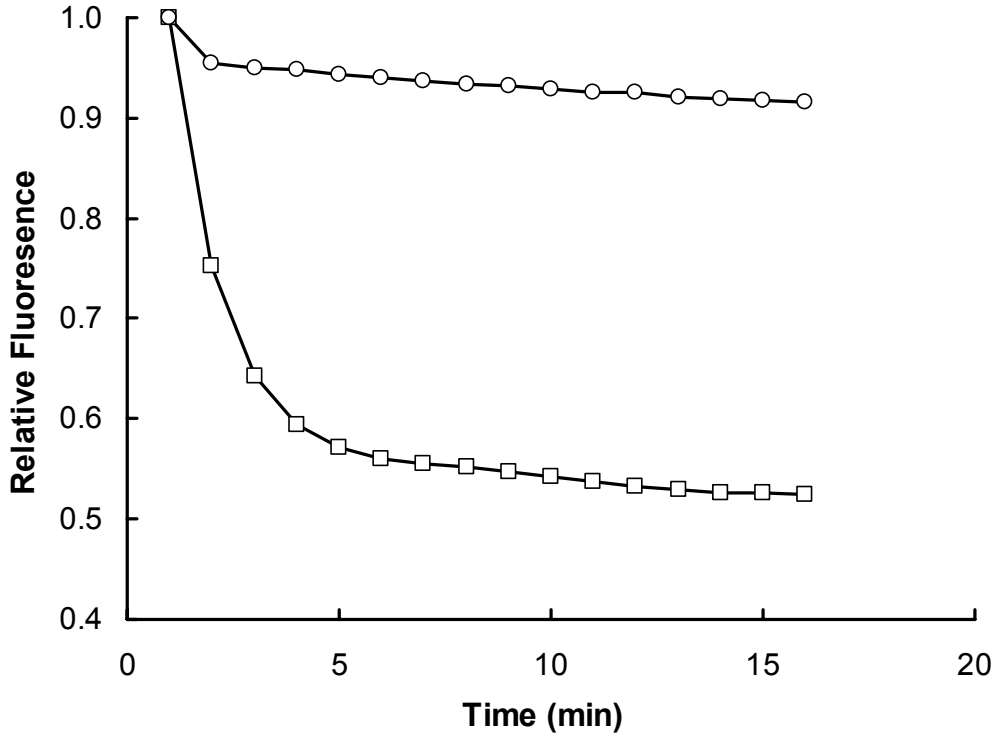

Figure 2.1. Fluorescein (FL) degradation by $^{\bullet}OH$ generated from Fe(III)/H_2O_2/ascorbic acid in different buffering systems. Reactions carried out in 96-well plates and fluorescence measured every minute using a plate reader with fluorescence detector with the following initial reaction concentrations: **(o)** 5.26×10^{-8} M FL, 42.5 mM sodium phosphate buffer (pH 7.4), 25 μM Fe(III), 26.5 mM H_2O_2, 100 μM ascorbic acid, 100 μM EDTA, 5% (v/v) acetone, final reaction pH remained 7.4; **(□)** 5.26×10^{-8}M FL, 30 mM potassium phosphate buffer (pH 7.4), 25 μM Fe(III), 26.5 mM H_2O_2, 100 μM ascorbic acid, 100 μM EDTA, 5% (v/v) acetone, final reaction pH was changed to 6.0

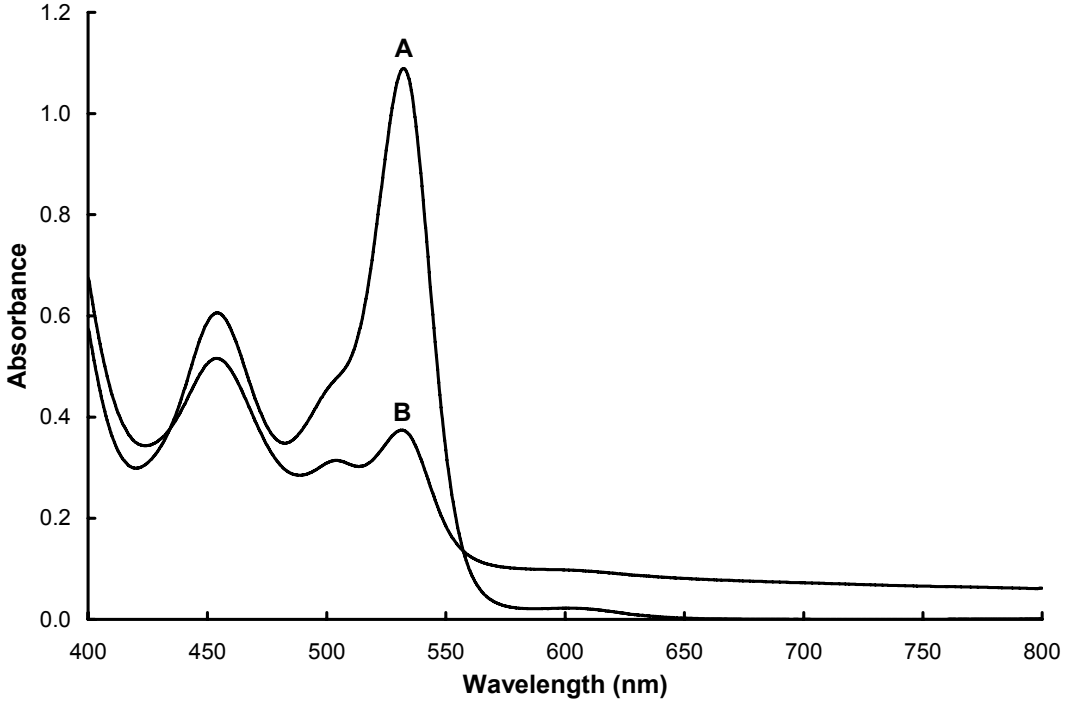

Figure 2.2. Absorption spectra of deoxyribose assay chromagen (A) without and (B) with acetone. Reaction conditions included the following initial reaction concentrations and conditions: 2.8 mM 2-deoxyribose, 200 μM $FeCl_3$ and 100 μM EDTA premixed, 10 mM potassium phosphate buffer at pH 7.4, 2.8 mM H_2O_2 prepared fresh, 5% (v/v) acetone or water as blanks, and 100 μM ascorbic acid made fresh. These reaction mixtures were heated for 60 minutes at 37ºC in a water bath followed by the addition of 1mL 2.8% trichloroacetic acid and 1 mL 1% thiobarbituric acid and heating in water bath at 80 ºC for 20 minutes. Reaction vessels then cooled in ice water bath for 15 minutes, centrifuged for 10 minutes, and absorbance spectra were recorded from 800 to 400 nm.

This study was undertaken to determine the optimal procedure for $^{\bullet}OH$ scavenging capacity assay under physiological pH. This method may meet the needs of antioxidant researchers in the food, nutrition, health, and medical fields.

2.3 Materials and Methods

Chemicals and Reagents. Fluorescein (FL), iron(III) chloride, iron(II) sulfate heptahydrate, copper(II) sulfate, cobalt(II) fluoride tetrahydrate, picolinic acid, disodium ethylenediaminetetraacetate (EDTA), 6-hydroxy-2,5,7,8-

tetramethylchroman-2-carboxylic acid (trolox), 5,5-dimethyl *N*-oxide pyrroline (DMPO), thiobarbituric acid, trichloroacetic acid, and ascorbic acid (Vit C) were purchased from Sigma-Aldrich. 30% H_2O_2 was purchased from Fischer Scientific. β-cyclodextrin (RMCD) was purchased from Cyclolab R & D Ltd. (Budapest, Hungary). Ultra-pure water was used for all experiments from an ELGA (Lowell, MA) Purelab Ultra Genetic polishing system with < 5ppb TOC and resistivity of 18.2mΩ. All other chemicals and solvents were of the highest commercial grade and used without further purification.

Reagent Preparation. A 1×10^{-5} M stock fluorescein solution was prepared in sodium phosphate buffer (75 mM pH = 7.4) and stored at 0ºC for future use. Stock fluorescein solution was diluted to 9.28×10^{-8} M with 75 mM sodium phosphate buffer (pH 7.4) fresh for each assay run. Iron(III) chloride, iron(II) sulfate, and copper(II) sulfate solutions were prepared daily in ultra-pure water. A cobalt solution was prepared daily by dissolving 15.7 mg of Co(II) fluoride tetrahydrate and 20 mg of picolinic acid in 10 mL of ultra-pure water. A 0.1990 M H_2O_2 solution was prepared fresh for each assay by diluting 30% H_2O_2 with ultra-pure water. A 7% (v/w) RMCD solution prepared was in ultra-pure water.

Natural Extracts Preparation. Extracts of natural materials with known antioxidant properties including cinnamon, hard wheat bran, and soft wheat grain were included in our in ESR experiments and our new HOSC assay to demonstrate their $^{\bullet}$OH scavenging capacities. Samples were ground to 20-mesh using a micro-

mill (Scienceware, Pequannock, NJ) and were extracted with 50% acetone for 24 hours in the dark, under nitrogen, at ambient temperature and pressure. All extracts were stored in the dark under nitrogen at room temperature.

Standards Preparation. Standard solutions of compounds with known radical scavenging activity including trolox, and *p*-coumaric, caffeic, ferulic, syringic and 4-OH benzoic acids were prepared in 50% acetone and stored in the dark under nitrogen. Trolox standards were prepared at the following concentrations, 20, 40, 60, 80, and 100 μM in 50% acetone for HOSC assay; 14, 44, 88, 116, and 160 μM in 50% acetone for the deoxyribose assay; and other concentrations in 50% acetone as indicated in figure captions.

HOSC Fluorometric $^{\bullet}$OH Scavenging Capacity Assay. Assay reactions were carried out in FluroNunc black 96-well polystyrene plates from Fischer Scientific and analyzed using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland) with an excitation wavelength of 485 nm, emission wavelength of 535 nm, and 0.1 second read time for each well with each plate read once per minute for 3 hours. The reaction mixtures contained 170 μL of 9.28×10^{-8} M FL prepared in 75 mM sodium phosphate buffer (pH 7.4), 30 μL of blank or extract or standard, 40 μL of 0.1990 M H_2O_2, and 60 μL of 3.43 mM $FeCl_3$, added in that order. Trolox concentrations of 20, 40, 60, 80, and 100 μM were used as standards. HOSC values were calculated using the regression equation between trolox concentration and net area under the FL decay curve as described by Ou and others (37). Area under the curve (AUC) values were calculated as

$$AUC = 0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \ldots + f_{i-1}/f_0 + 0.5(f_i/f_0)$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the final fluorescence reading. The net AUC is calculated by subtracting the AUC of the blank from the AUC of the sample. Relative HOSC values were expressed as μmoles of trolox equivalents (TE) per gram material, and μmoles of TE per μmole compound for pure compounds, with both calculations shown below.

Relative HOSC value for pure compounds = $[(AUC_{sample} - AUC_{blank}) / (AUC_{trolox} - AUC_{blank})] \times$ (molarity of trolox / molarity of sample), and

Relative HOSC value for natural extracts = $[(AUC_{sample} - AUC_{blank}) / (AUC_{trolox} - AUC_{blank})] \times$ (molarity of trolox / concentration of sample).

Deoxyribose Assay. Deoxyribose assay conducted according to a previously described protocol (*17*). Initial reaction concentrations were 2.8 mM 2-deoxyribose, 200 μM $FeCl_3$ and 100 μM EDTA premixed, 10 mM potassium phosphate buffer at pH 7.4, 2.8 mM H_2O_2 prepared fresh, 5% (v/v) acetone as blank, and 100 μM ascorbic acid made fresh. The total volume was 1.2 mL for all reaction mixtures. The reaction mixtures were kept for 60 minutes at 37 ºC followed by the addition of 1 mL 2.8% trichloroacetic acid and 1 mL 1% thiobarbituric acid and heating in water bath at 80ºC for 20 minutes. Reaction vessels then cooled in ice water for 15 minutes, centrifuged for 10 minutes, and absorbance read at 532 nm with a Genesys 20 spectrophotometer (Thermo Spectronic, Rochester, NY). Absorption spectra were taken with a UV-1601 spectrophotometer (Shimadzu, Columbia, MD).

Fluorescein Degradation Using Deoxyribose $^{\bullet}OH$ generating System Under Two pH's. Experimental conditions included $^{\bullet}OH$ generating system reaction used in deoxyribose assay (*17*) with fluorescein probe. Final reaction pH 7.4 experiment included the following initial reaction concentrations: 5.26×10^{-8} M FL, 42.5 mM sodium phosphate buffer (pH 7.4), 25 μM $FeCl_3$ 26.5 mM H_2O_2, 100 μM ascorbic acid, 100 μM EDTA, 5% (v/v) acetone. Final reaction pH 6.0 experiment carried out with same conditions except buffering system which was 30 mM (initial reaction concentration) potassium phosphate buffer (pH 7.4). Fluorescence measurements made using the same equipment and conditions as used in the HOSC assay.

Oxygen Radical Absorbance Capacity (ORAC) Assay. 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 (*37*). Standards were prepared in 50% acetone 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 standard or 50% acetone for blanks, and 25 μL of 0.6 M AAPH, and for blank. The fluorescence of the assay mixture was recorded every minute for 2 hours at ambient temperature. 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 (*38*) with results expressed as μmoles of TE per gram material for natural extracts, and μmoles of TE per μmole compound for pure standards.

ESR Spin Trapping Assay. ROS generation and $^{\bullet}OH$ scavenging were examined by an ESR method using DMPO as the trapping agent. The reaction mixtures contained 75 μL 85 mM sodium phosphate buffer (pH 7.4), 10 μL of 1.5 M DMPO, 15 μL of blank or extract or standard, 20 μL 0.1990 M H_2O_2, and 30 μL of 3.43 mM $FeCl_3$, added in that order. Initial reaction concentrations were 42.5 mM sodium phosphate buffer, 26.53 mM H_2O_2, 0.686 mM $FeCl_3$, and 10% (v/v) sample extract or solvent blank. All reaction mixtures were vortexed and held 2 minutes before analysis using a Varian E-109X-Band ESR spectrometer (Varian, Inc., Palo, CA). Conventional ESR spectra were recorded with 15 mW incident microwave power and 100 kHz field modulation of 1G at ambient temperature. Under testing conditions, $^{\bullet}OH$ signal strength was proportional to its concentration.

Statistical Analysis. Data were reported as mean ± SD for triplicate determinations. ANOVA and Tukey's tests were performed (SPSS for Windows, Version Rel. 10.0.5, 1999, SPSS Inc., Chicago, IL) to identify differences among means. A two-tailed Pearson's correlation test was conducted to determine the correlations among means. Statistical significance was declared at $P \leq 0.05$.

2.4 Results and Discussion

It is widely accepted that an ideal radical scavenging activity assay should have the following characteristics: 1) simple, 2) produces of a steady flux of pure radicals throughout the assay timeframe from a biologically relevant radical generating system, 3) utilizes a physiological reaction pH, 4) utilizes equipment commonly

available in antioxidant research labs, 5) has a definite endpoint and chemical mechanism, 6) is compatible with commonly used extraction solvents, 7) is validated for performance characteristics, and 8) is adaptable to "high-throughput" analysis (*14*). In the present study, a novel fluorometric procedure was developed and validated for $^{\bullet}$OH scavenging capacity estimation.

$^{\bullet}$OH Generating System Selection. Four $^{\bullet}$OH generating systems were evaluated for their capacities to generate a steady flux of pure $^{\bullet}$OH under physiological pH, using fluorescein (FL) as the ROS detecting probe. These systems included the superoxide driven Fenton reaction (*25*) and "Fenton-like" reagents including Co(II)/H_2O_2 (*26*), Cu(II)/H_2O_2 (*27, 28*), and Fe(III)/H_2O_2, a system that has been shown to cause DNA stand breakage (*39*) and presumably produce $^{\bullet}$OH (*40*) at physiological pH. The Co(II)/H_2O_2, Cu(II)/H_2O_2, and Fe(III)/H_2O_2 systems caused a significant drop in FL fluorescence indicating the presence of ROS and their attack on FL, while the superoxide-driven Fenton system under the experimental conditions caused no degradation to FL at pH 7.4, suggesting little ROS formation (**Figure 2.3**). These results suggested that Co(II)/H_2O_2, Cu(II)/H_2O_2, and Fe(III)/H_2O_2 systems may be suitable choices for $^{\bullet}$OH scavenging capacity assays.

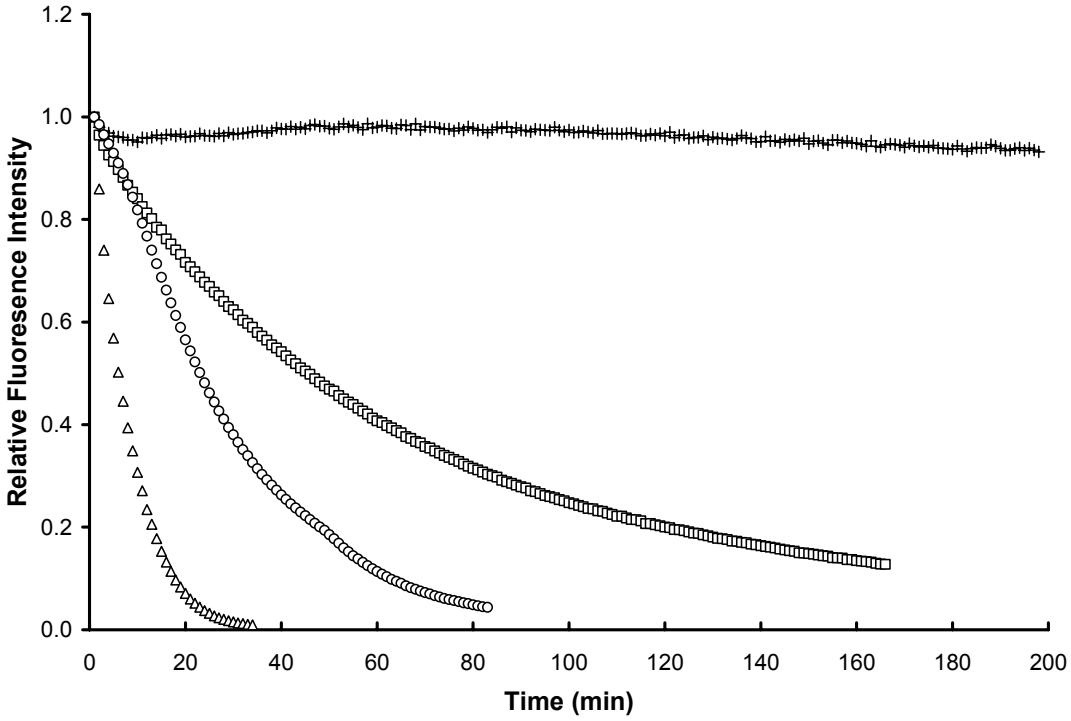

Figure 2.3. Fluorescent decay curves of fluorescence with selected Fenton-like reaction systems. Fluorescence decay curve of fluorescein were prepared to compare and evaluate different proposed Fenton-like hydroxyl radical generating systems. All reactions were performed in 96-well plates with the initial reaction concentrations of 42.5 mM phosphate buffer (pH 7.4), 5.26×10^{-8} M FL, and 5% (v/v) acetone, added in that order, followed by: **(□)** 0.343 mM Fe(III), 26.5 mM H_2O_2; **(Δ)** 0.95 mM Cu(II), 26.5 mM H_2O_2; **(+)** 0.08 mM Fe(II), 0.08 mM EDTA **(o)** 230 μM Co(II), 2.75×10^{-2}M H_2O_2.

It is widely accepted that Co(II), Cu(II), and Fe(III), in combination with H_2O_2, called "Fenton-like" reagents generate ROS, but the exact species formed and their mechanisms have been greatly debated in literatures (*41, 42*). Further investigations were conducted with the Co(II)/H_2O_2, Cu(II)/H_2O_2, Fe(III)/H_2O_2 systems to verify the ROS generated under the experimental conditions with ESR using DMPO as the spin trapping agent. The time-dependent ESR spectra of DMPO spin adducts formed during the Co(II)/H_2O_2 reaction (**Figure 2.4a)** showed a mixture of DMPO/$^{\bullet}$OH and possibly DMPO/$O_2^{\bullet-}$ adducts formed at pH 7.4 (*43*). These results were supported by

the conclusions of Kadiiska and others (*44*) who found a mixture of $^{\bullet}OH$ and $O_2^{\bullet-}$ generated by this system at physiological pH, and others who have proposed that ROS besides $^{\bullet}OH$ could be generated by this system at physiological pH (*45* - *47*). Similarly for the Cu(II)/H_2O_2 reaction, a mixture of DMPO/$^{\bullet}OH$ and possibly DMPO/$O_2^{\bullet-}$ adducts were observed in the time-dependent ESR spectra of DMPO adducts formed at pH 7.4 (**Figure 2.4b**). This observation was supported by the previous finding of ROS besides $^{\bullet}OH$ generated by the Cu(II)/H_2O_2 system at physiological pH (*48* - *50*). The results for both the Co(II)/H_2O_2 and Cu(II)/H_2O_2 systems indicated that they would not produce a steady flux of pure $^{\bullet}OH$, and therefore were not suitable for a $^{\bullet}OH$ scavenging capacity estimation assay.

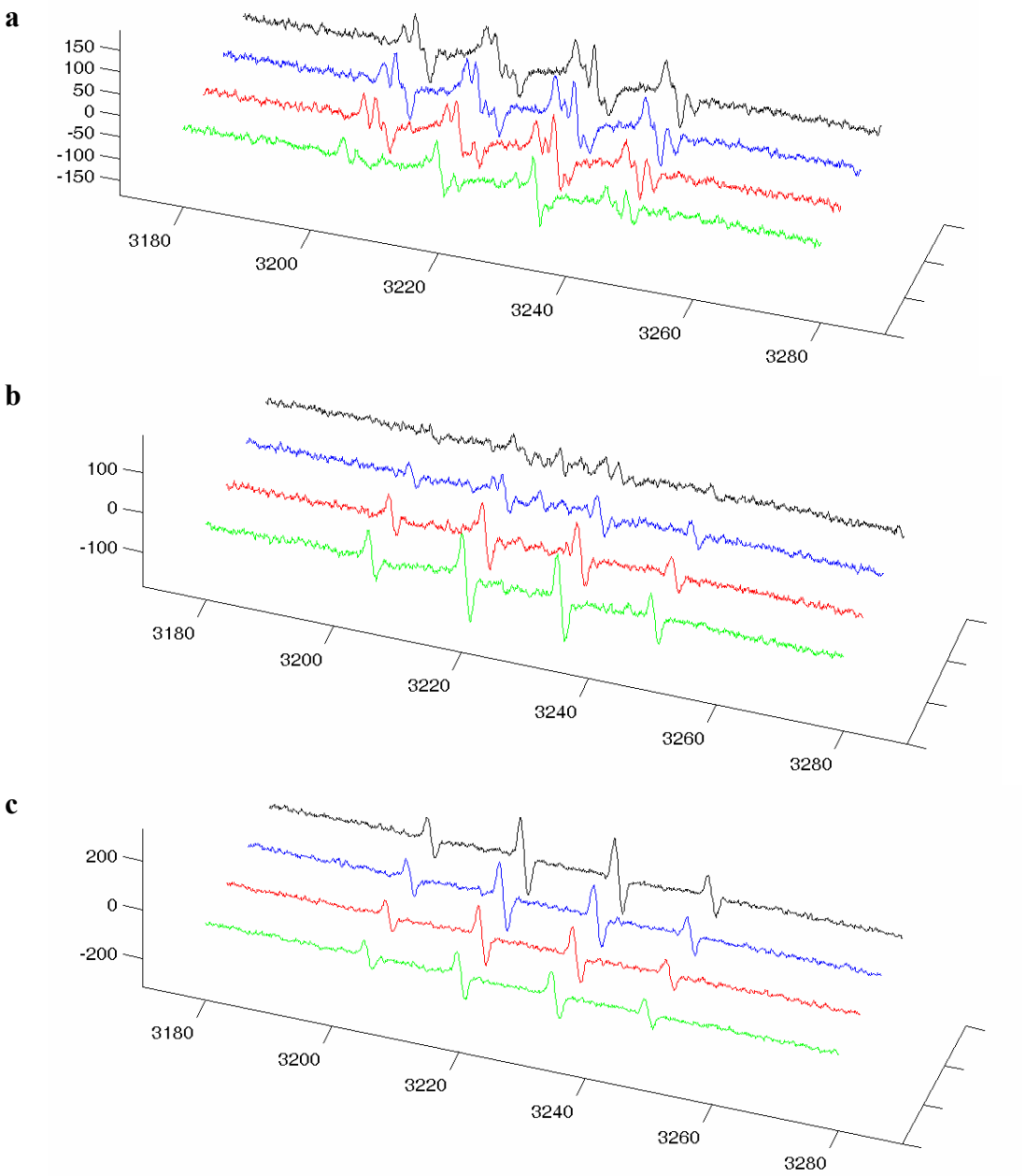

Figure 2.4. Reactive oxygen species generated by the (a) Co(II)/H_2O_2, (b) Cu(II)/H_2O_2, and (c) Fe(III)/H_2O_2 Fenton-like reaction system. Reactive oxygen species were determined with an ESR method using DMPO as the spin trapping agent with the following initial reaction concentrations: (a) 42.5 mM sodium phosphate buffer (pH 7.4), 0.1 M DMPO, 230 µM Co(II), 2.75×10^{-2}M H_2O_2, 5% (v/v) acetone; (b) 42.5 mM sodium phosphate buffer (pH 7.4), 0.1 M DMPO, 0.95 mM Cu(II), 26.5 mM H_2O_2, 5% (v/v) acetone; (c) 42.5 mM sodium phosphate buffer (pH 7.4), 0.1 M DMPO, 0.686 mM Fe(III), 26.5 mM H_2O_2, 5% (v/v) acetone. Measurements taken at 0, 9, 18, and 30 minutes are indicated by the colors green, red, blue, and black, respectively. Spectra in (c) shows typical hydroxyl radical quartet signal with an intensity ratio of 1:2:2:1 ($a_N = a_H = 14.7$ G).

The time-dependent ESR spectra of DMPO spin adducts formed during the Fe(III)/H_2O_2 reaction at pH 7.4 are shown in **Figure 2.4c**. This figure shows a typical DMPO/$^{\bullet}$OH adduct signal with characteristic quartet signal intensity ratio of 1:2:2:1 and hyperfine coupling $a_N = a_H = 14.7$ G (*51*) which is consistently present throughout the entire 30 minute testing time-frame. This indicated that the tested Fe(III)/H_2O_2 system with no chelating agent was capable of producing a steady flux of pure $^{\bullet}$OH, making it an ideal $^{\bullet}$OH generating system for a $^{\bullet}$OH scavenging capacity assay. While ferrous and not ferric iron, is the oxidation state of iron typically associated with the classic Fenton reaction, numerous researchers have reported the generation of ROS from the ferric reaction including Tachon (*39*) who reported DNA degradation in a buffered system, and Kocha and others (*40*) who reported aromatic hydroxylation of benzoate using EDTA chelated ferric iron and H_2O_2 in pH 7.2 buffered system. In addition, other researchers (*52 – 54*) have reported the generation of $^{\bullet}$OH from chelated ferric iron/H_2O_2, using ESR with DMPO as a spin trapping agent. The mechanism of this reaction and even the classic Fenton has been debated over the years and is still not completely understood (*55*). The complexity in analyzing these mechanisms is attributed to the competition of mechanisms depending on reaction conditions such as pH, solvents, ligands, the presence of oxidizable organic substrates, and the extremely short lives of many of the reactive species (*56*).

A commonly accepted mechanism for this reaction involves a radical chain reaction with Fe(II), $HO^{\bullet}$, $O_2^{\bullet-}$, and $^{\bullet}$OOH as propagating species and was originally

proposed by Barb and others (*57*) often referred to as the superoxide or Haber Weiss driven Fenton reaction. This reaction was originally thought to involve the direct oxidation of Fe(III) by H_2O_2 to form $O_2^{\bullet-}$ under basic conditions or $^{\bullet}OOH$ under acidic conditions, but was later hypothesized to involve an intermediate ferric-hydroperoxy complex (*53*). This mechanism under acidic conditions outlined by Perez-Benito (*58*) begins with the reaction of Fe(III) and H_2O_2 to form an intermediate, the ferric-hydroperoxy complex $(Fe(III)\text{-}OOH]^{2+}$, which decomposes to Fe^{2+} and $^{\bullet}OOH$, with ferrous iron reacting with H_2O_2 to yield $^{\bullet}OH$. This mechanism has been supported by De Laat and Gallard's kinetic study (*59*). Ensing and others (*56*), however, using DFT calculations and Car-Parrinello molecular dynamic simulations has shown that the probable fate of the $[Fe(III)\text{-}OOH]^{2+}$ intermediate is not the homolysis of the Fe-O bond to form Fe^{2+} and $^{\bullet}OOH$, but the homolysis of the O-O bond to form $[Fe^{IV}O]^{2+}$ and $^{\bullet}OH$. Our ESR study results (**Figure 2.4c**) showing no detectable $O_2^{\bullet-}$ are in agreement with Ensing and others (*56*), supporting the homolysis of the O-O bind to form $[Fe^{IV}O]^{2+}$ and $^{\bullet}OH$ under the experimental conditions.

In addition to the advantage of generating pure $^{\bullet}OH$, the ferric iron/H_2O_2 system has physiological relevance. Ferric iron is predominately found in the body bound to ferritin and tranferritin proteins. It also likely exists in the free iron pool, also referred to as "labile iron pool," as ferric iron chelated by biological chelators such as ADP, ATP, GTP or citrate. This "free" iron pool is thought to be available for pathological free radical reactions (*60*). *In-vivo* studies demonstrated that rats

exposed to Fe(III) chelated with NTA caused renal carcinoma and induced DNA lesions (*1*), supporting the role of Fe(III) in oxidative reaction under physiological conditions. Given its physiological relevance and superior $^{\bullet}OH$ generating properties under physiological pH found in this study and supported by the conclusions of other researchers, the ferric iron/H_2O_2 was chosen as the $^{\bullet}OH$ generating system for the new assay.

Detection Probe Selection. Numerous detection probes, another critical component of free radical scavenging assays, have been developed for monitoring $^{\bullet}OH$ concentration and $^{\bullet}OH$ scavenging capacity. These probes, including 2-deoxyribose/"TBA-reactive" substances (*17*), DNA (*15*), methionine (*58*); aromatic compounds (*15*) such as salicylic acid (*23*), DMPO adducts (*20*), fluorescamine-derivatized nitroxide (*21*), fluorescein (*26*), β-Phcoerythrin (*27*), coumarin derivatives (*28*); and the chemiluminescent probe, luminal (*19*), are used for different detection methods such as molecular absorbance and fluorescence, GLC, ESR, MS or chemiluminescence, in possible combination with separation technologies including GLC, GC, and HPLC (*15*). The ESR spin trapping methods have excellent sensitivity and specificity, but are often not carried out under biological relevant conditions such as pH (*62*), and require expensive equipment (*63*). Chemiluminescence methods, though sensitive, utilize a transient signal which is not ideal for scavenging capacity assays, and don't have good specificity for $^{\bullet}OH$ (*25*). MS detections require expensive instruments and are difficult to implement. This left fluorescence and molecular absorption as good potential detection methods with many potential

probes.

Fluorescein was chosen as the detection probe for the new hydroxyl radical scavenging capacity (HOSC) assay because of its high sensitivity to $^{\bullet}OH$ attack, high quantum yield, no interferences with phenolic compounds, its photostability, stability under physiological pH, its wide acceptability in radical scavenging capacity assays such as the popular ORAC method (*38*), its availability and reasonable cost.

Evidence of Definite Endpoint for HOSC. A definite endpoint is critical for quantification of $^{\bullet}OH$ scavenging capacity. The fluorescence decay curves of fluorescein with different trolox concentrations were determined using the ferric iron/H_2O_2 $^{\bullet}OH$ generating system in pH 7.4 phosphate buffer (**Figure 2.5**). Trolox is a known free radical scavenging compound which is a commonly used antioxidant standard in other free radical scavenging capacity assays including the popular ORAC. The decay curves clearly showed a dose-dependent lag phase inhibition of trolox, similar to that observed for trolox in the ORAC assay (*38*), indicating the potential of this new assay in quantifying the $^{\bullet}OH$ scavenging capacity of antioxidant preparations.

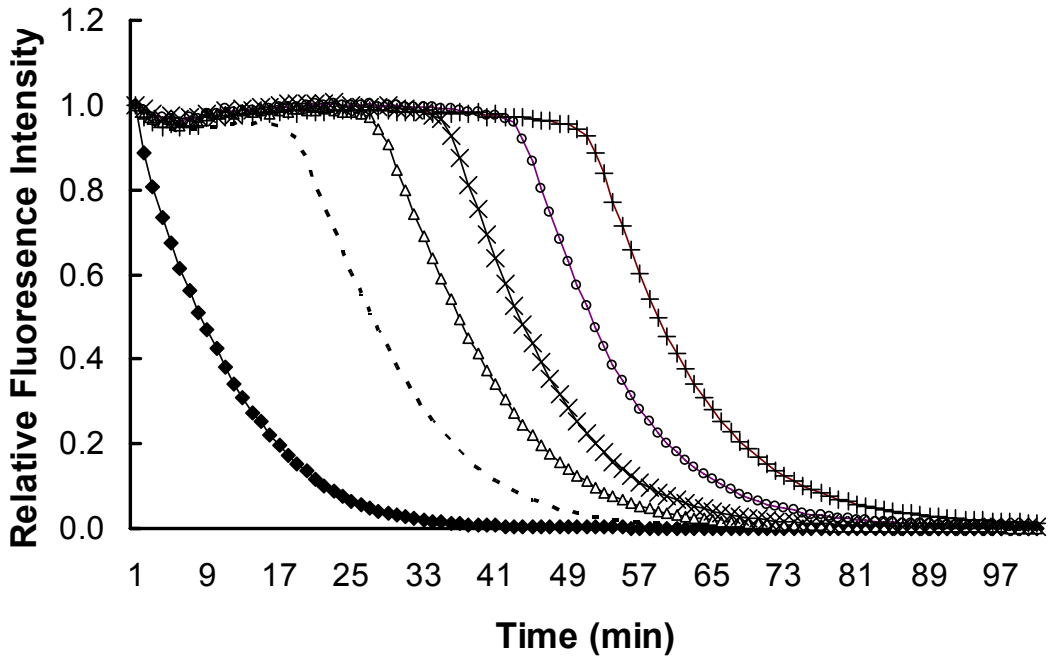

Figure 2.5. Fluorescence decay curve of fluorescein in the presence of trolox under the HOSC assay condition. Initial concentrations in the reaction mixtures were 5.26×10^{-8}M FL, 42.5 mM sodium phosphate buffer, 26.53 mM H_2O_2, 0.686 mM $FeCl_3$, and 10% (v/v) trolox solution or blank prepared in 50% acetone at the following concentrations: **(■)** blank containing no trolox, **(-)** 20 μM, **(Δ)** 40 μM, **(x)** 60μM, **(o)** 80 μM, and **(+)** 100 μM trolox, respectively.

Scavenging activities for the HOSC assay are calculated using the net area under the curve (AUC) as used in other established assays such as ORAC (38). This method for calculating HOSC takes into accounts both the inhibition time and extent, and utilizes a definite endpoint. This is especially important as antioxidants have complex kinetics, some exhibiting a lag phase while others do not. This AUC calculation method is therefore viewed as being superior to percent inhibition or fixed time point calculations (*14, 64*).

Confirmation of HOSC Scavenging Measurement Against Pure $^{\bullet}$OH. To verify that the HOSC assay does measure scavenging activity against pure $^{\bullet}$OH, an

ESR spin trapping assay was performed with both antioxidant standards and natural extracts with results shown in **Figure 2.6**. These ESR results clearly show scavenging activities of these compounds and extracts, and the presence of only $^{\bullet}$OH in the reaction mixtures (**Figure 2.6**). Furthermore, these ESR results showed an order of the $^{\bullet}$OH scavenging activity similar to that obtained by the HOSC method (**Figure 2.6, Figure 2.7, Table 2.1**).

Table 2.1. HOSC Values for Natural Extracts

Food Extract	HOSC (TE)[a]	SD[b]
soft wheat	38.78	7.19
hard wheat bran	74.91	8.18
cinnamon	3009.85	233.72

[a] TE, trolox equivalents expressed as μmoles trolox per g material.

[b] SD, Standard deviation

Correlation Between HOSC and ORAC Values. The HOSC and ORAC values of the five selected phenolic acids are presented in **Figure 2.7**. The greater value of trolox equivalents (TE) per μmole of phenolic acid is associated with a stronger HOSC or ORAC. Strong correlation ($r = 0.954$,$P = 0.05$) was observed between HOSC and ORAC values of the five antioxidative phenolic acids. Also noted was that the trend seen from these two assays in **Figure 2.7** agrees with results from Zhou and others (*65*) who studied the $^{\bullet}$OH scavenging activities of coumaric, ferulic, syringic, and vanillic acids using ESR spin trapping method and found coumaric acid to have the strongest activity.

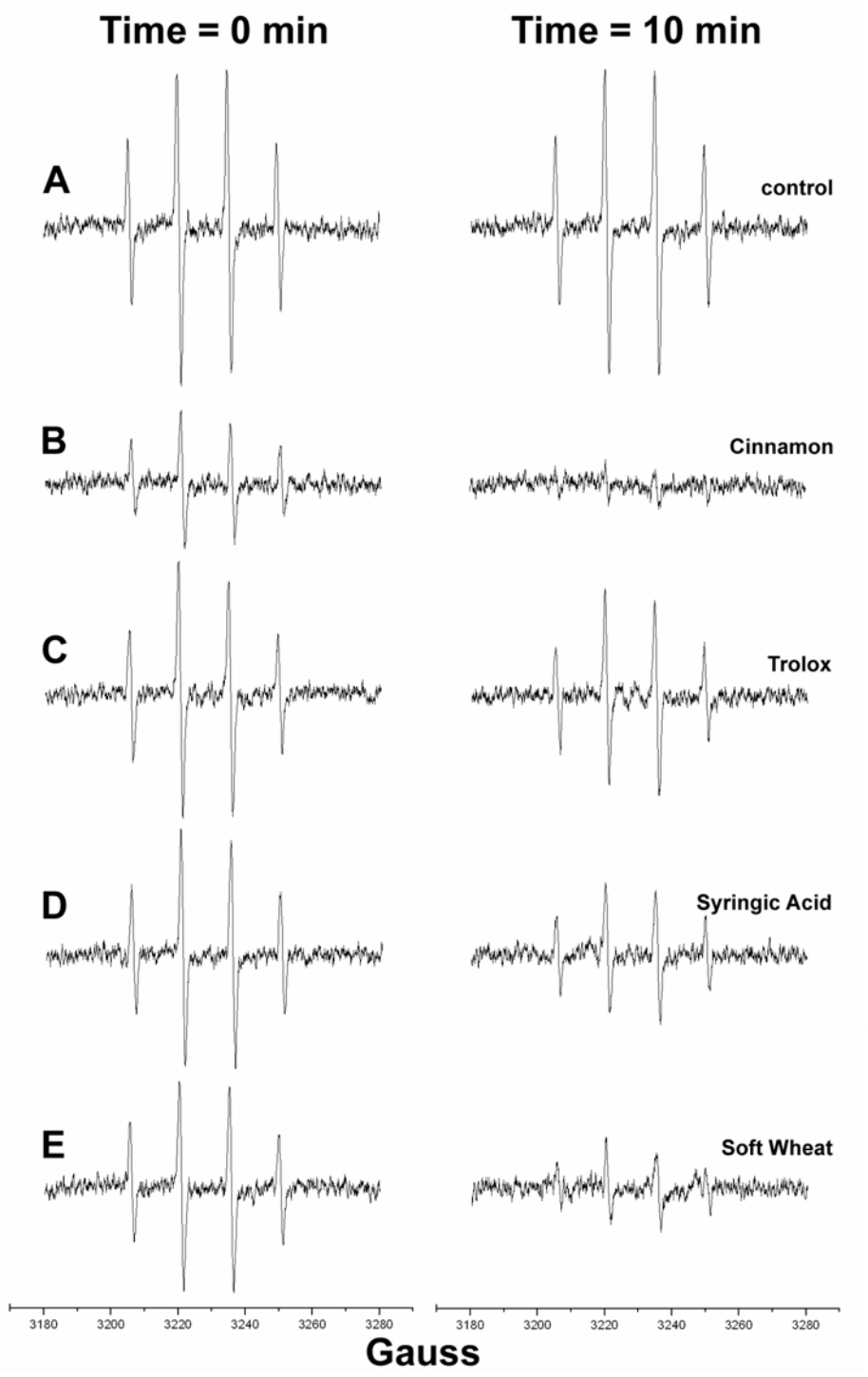

Figure 2.6. Scavenging activity of antioxidants and natural extracts using ESR DMPO spin trapping assay with Fe(III)/H_2O_2. Initial reaction concentrations were: 42.5 mM sodium phosphate buffer (pH 7.4), 0.1 M DMPO, 0.686 mM Fe(III), 26.5 mM H_2O_2, 10% (v/v) standards or extracts all prepared in 50% acetone as follows: (A) blank, (B) 0.1mg/mL cinnamon extract, (C) 20 mM trolox, (D) 20 mM syringic acid, (E) 0.1 mg/mL whole grain soft wheat extract. Measurements were taken at 0 and 10 minutes.

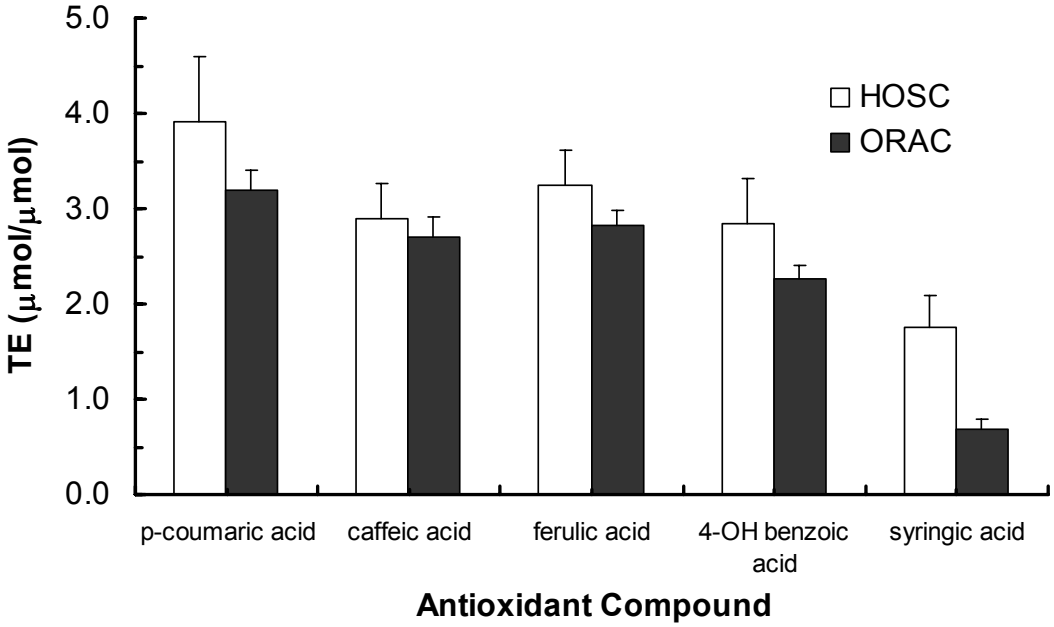

Figure 2.7. HOSC and ORAC values of five phenolic acids. Initial reaction concentrations for HOSC assay were 5.26×10^{-8}M FL, 42.5 mM sodium phosphate buffer, 26.53 mM H_2O_2, 0.686 mM $FeCl_3$, and individual phenolic acids at 2.5 μM. Initial reaction concentrations for ORAC assay were 0.067 μM FL, 53.6 mM AAPH, and individual phenolic acids at 2.7 μM. Results were reported as μmoles trolox equivalents (TE) per μmole of phenolic acid. All tests were conducted in triplicates with mean values reported. The vertical bars represent the standard deviation of each data point (n = 3).

Effect of Extraction Solvent on HOSC. One of the criteria for a potential assay was compatibility of the assay with different solvents. The effects of commonly used extraction solvents including acetone, ethanol, methanol, DMSO, and these in combination with β-cyclodextrin (RMCD) were evaluated using the HOSC assay and results are shown in **Figure 2.8**. RMCD was included because of its application as solubility enhancer for antioxidant activity evaluation in aqueous phase. Results indicated that 50% acetone, a commonly used solvent for extracting phenolics (*37*), had almost no effect on the kinetics of FL degradation by $^{\bullet}$OH, while the remaining solvents (ethanol, methanol, and DMSO) all showed very different kinetics with

almost no FL degradation, suggesting that acetone has little interference with HOSC assay and aqueous acetone is a better choice of solvent for HOSC determination. Furthermore, adding RMCD to 50% acetone significantly slowed the degradation of FL compared to 50% acetone without RMCD. The effect of RMCD on the degradation of FL for the remaining solvents showed no effect for DMSO and a slight increase for ethanol and methanol.

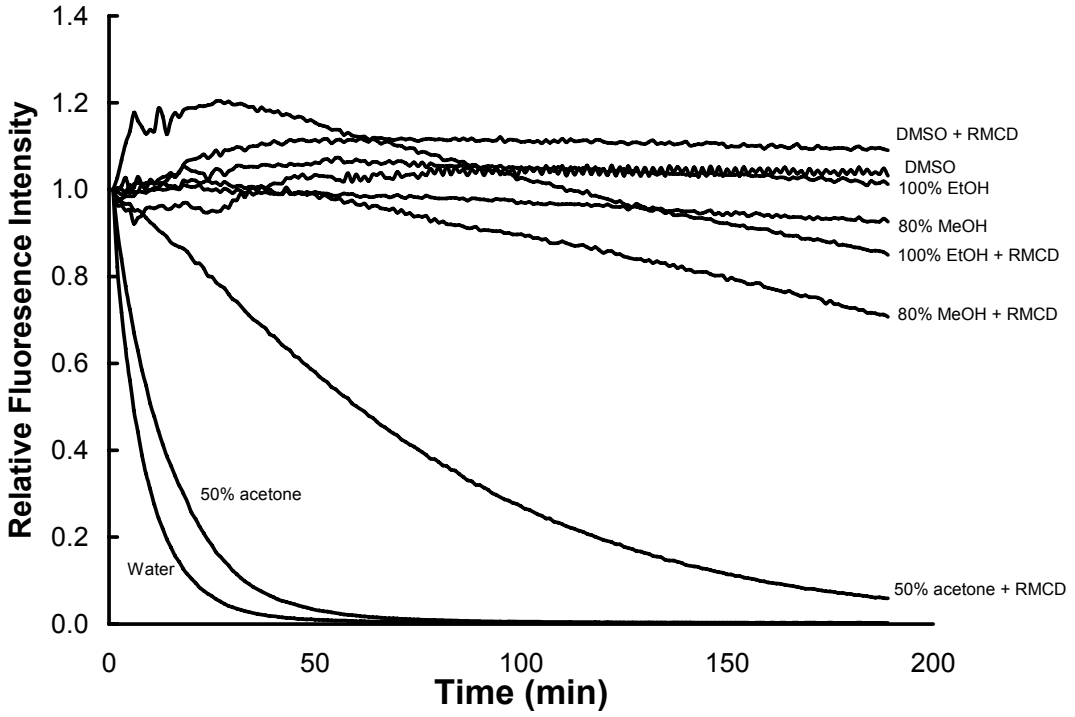

Figure 2.8. Effect of organic solvents and β-cyclodextrin on fluorescein degradation in HOSC assay. Initial reaction concentrations for HOSC assay were 5.26×10^{-8}M FL, 42.5 mM sodium phosphate buffer, 26.53 mM H_2O_2, 0.686 mM $FeCl_3$, and 10% (v/v) of the following solvents: water, 50% acetone, 80% methanol (MeOH), 100% ethanol (EtOH), 100% dimethyl sulfoxide (DMSO), and the above with β-cyclodextrin at 1.4% (w/v) (RMCD).

To further understand the solvent effect on the HOSC assay, an ESR spin trapping

assay with DMPO was conducted. ESR spectra of DMPO adducts (**Figure 2.9)** indicated the formation of carbon centered radicals when ethanol, methanol, and DMSO were included in the HOSC assay**,** while $^{\bullet}OH$ was the only radical in the testing system containing acetone. These carbon centered radicals are likely formed due to proton abstraction from ethanol, methanol, and DMSO. These results were expected as ethanol, methanol, and DMSO are known to be excellent $^{\bullet}OH$ scavengers, and are known to form carbon centered radicals such as α-hydroxyethyl and hydroxymethyl radicals from ethanol and DMSO or methanol respectively which can be spin trapped and are often used as detector molecules in $^{\bullet}OH$ assays (*15, 64*). These results also further support the earlier notion that the Fe(III)/H_2O_2 system is a source $^{\bullet}OH$ and not $O_2^{\bullet-}$ as the latter does not exhibit these properties (*66*).

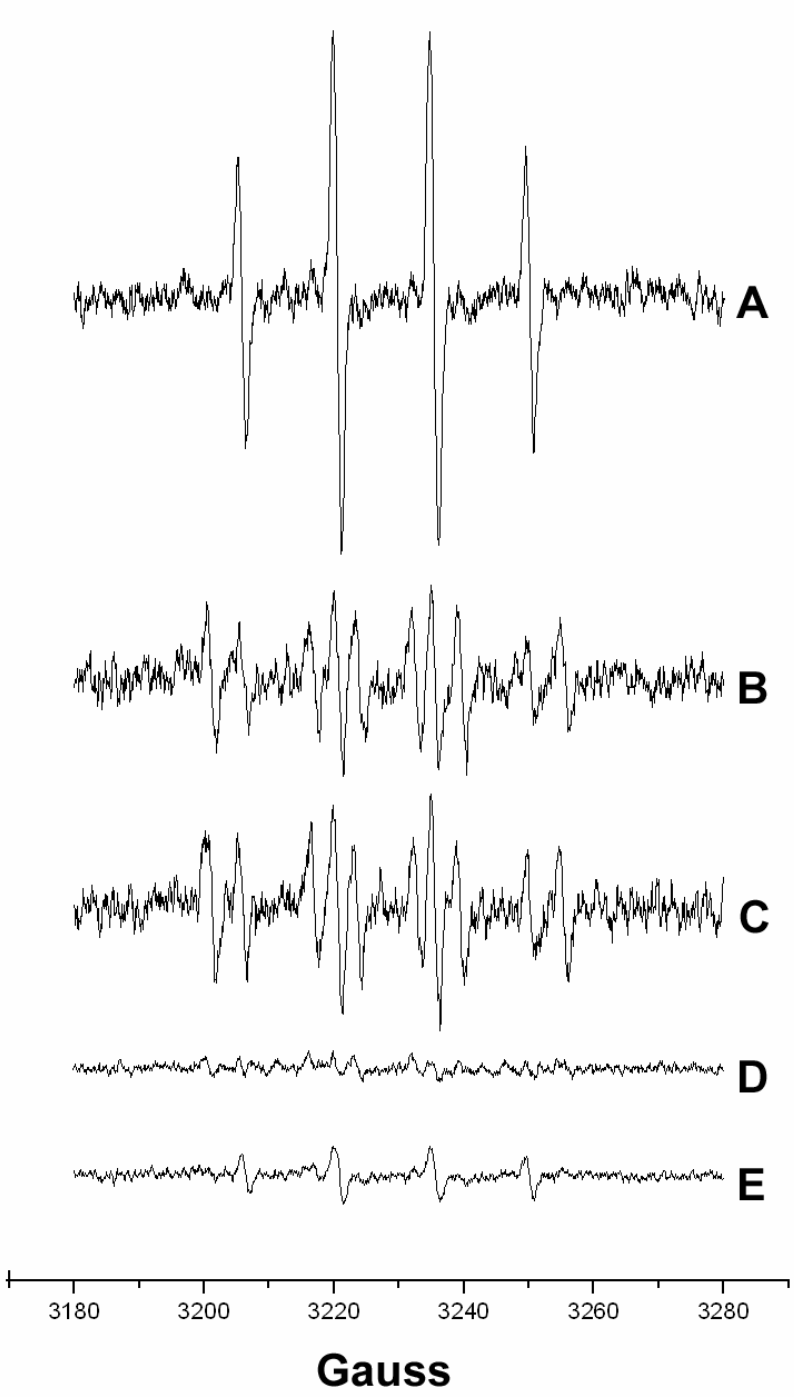

Figure 2.9. ESR spectra of DMPO-ROS adducts observed in the presence of different solvents and β-cyclodextrin using Fe(III)/H_2O_2 as $^{\bullet}OH$ generating system. Initial reaction concentrations were 42.5 mM phosphate buffer (pH 7.4), 0.1M DMPO, 0.686 mM Fe(III), 26.5 mM H_2O_2, and 10% (v/v) of the following solvents: (A) 50% acetone (B) 100% ethanol (EtOH), (C) 80% methanol (MeOH), (D) 100% dimethyl sulfoxide (DMSO), (E) 50% acetone and 1.4% (w/v) β-cyclodextrin (RMCD).

The effect of RMCD in combination with acetone was also investigated with the ESR using DMPO as the spin trapping agent, and results showed a significant decrease in the signal intensity of DMPO/$^{\bullet}$OH adduct with RMCD indicating that RMCD was able to inhibit the generation of $^{\bullet}$OH under the experimental conditions or that it may react with and quench $^{\bullet}$OH with a possibility of forming radicals that were not trappable with DMPO (**Figure 2.9**). These results confirm the interference of RMCD on HOSC measurements, which may reduce the sensitivity of HOSC measurements. In summary, only water and acetone are compatible with the HOSC assay. It should be pointed out that these solvent compatibility issues should be expected for any $^{\bullet}$OH assay and not only for the HOSC, because of the chemical nature of $^{\bullet}$OH and these solvents.

Linearity and Range of HOSC Assay. A linear relationship between net area under the fluorescence decay curve (AUC) and antioxidant concentration is important for HOSC value measurement. The linear relationship for a group of pure antioxidant compounds and botanical extracts including trolox, ferulic acid, *p*-coumaric acid, soft wheat extract, and cinnamon extract, all known free radical scavengers, is shown in **Table 2.2**. Trolox was chosen as the antioxidant standard to express other antioxidants for this assay because of its excellent linear relationship (R^2=0.99) and sensitivity (slope = 0.4) under the experimental conditions, and its wide use as a standard in other assays such as ORAC. HOSC values were therefore expressed as trolox equivalents with units of trolox equivalents (TE) per unit of testing sample. The linear range for trolox was found between 20 and 100 μM in the testing solution.

Table 2.2. Linear Relationships Between Antioxidant Concentration and Net Area Under the Curve using HOSC assay*

Compound	Concentration Range	R^2	Slope	Y-Intercept
Trolox	20 – 100 μM	0.993	0.400	9.595
P-coumaric acid	2 – 40 μM	0.995	1.671	0.928
Ferulic acid	2 – 60 μM	0.994	1.369	6.201
Soft wheat	0.770 – 2 μg/mL	0.968	22.182	0.177
Cinnamon	83.3 – 200 ng/mL	0.979	462.430	30.808

*All measurements conducted under HOSC assay conditions with antioxidant and botanical extracts in 50% acetone. All tests conducted in triplicate.

Reproducibility, Accuracy and Precision of HOSC assay. The day to day reproducibility of the HOSC method was assessed using a 25 μM caffeic acid standard measured 6 times in triplicate over 12 days. Results showed an average TE value of 4.16 and percent relative standard deviation (%RSD) of 4.06% during the testing time frame **(data not shown)** suggesting that the HOSC procedure is stable for day to day measurements. The accuracy and precision of the method were evaluated by comparing three runs of trolox at three concentrations within a day. **Table 2.3** summarizes the results and indicates a pooled precision measured as percent relative standard deviation (%RSD) within ± 15% and pooled accuracy measured as percent recovery (% REC) between 96 and 104%.

Table 2.3. Precision and Accuracy of Quality Control (QC) Samples

	QC1	QC2	QC3
nominal [trolox] (μM)	60	80	100
run 1			
intra-mean (μM)	61.58	81.82	96.63
SD[a]	2.36	11.02	10.45
% RSD[b]	3.84	13.47	10.82
% REC[c]	102.63	102.27	96.63
n	3	3	3
run 2			
intra-mean (μM)	60.83	85.50	94.91
SD[a]	5.26	6.79	10.31
% RSD[b]	8.65	7.94	10.87
% REC[c]	101.39	106.87	94.91
n	3	3	3
run 3			
intra-mean (μM)	62.82	81.58	96.91
SD[a]	1.63	8.48	8.71
% RSD[b]	2.60	10.40	8.98
% REC[c]	104.70	101.97	96.91
n	3	3	3
pooled runs			
inter-mean (μM)	61.74	82.97	96.15
SD[a]	3.12	7.97	8.59
% RSD[b]	5.06	9.60	8.93
% REC[c]	102.90	103.71	96.15
n	9	9	9

[a] SD, standard deviation

[b] % RSD, percent relative standard deviation

[c] % REC, percent recovery

2.5 Conclusion

A novel hydroxyl radical scavenging capacity assay named HOSC was described and validated. The HOSC assay measures the scavenging capacity of antioxidant samples against a constant flux of pure hydroxyl radicals under physiological pH, with a definite end point. This method uses an inexpensive stable common fluorescent probe and may be performed with a plate reader with a fluorescence detector for high-throughput analyses or scaled up and adapted to a single read fluorometer. The HOSC assay is compatible with water and acetone extracts and has excellent correlation with a popular radical scavenging assay (ORAC). Finally, the HOSC assay has acceptable sensitivity, ruggedness, precision, and accuracy. Compared to the commonly used deoxyribose method, the HOSC method has better compatibility with acetone, and more efficiently generates hydroxyl radicals under physiological pH. The HOSC method may serve as an important tool for those researchers interested in the radical scavenging capacities of both food extracts and pure antioxidant compounds with applications in the food, biological, and medical fields.

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Chapter 3: Effects of Solid-State Enzymatic Treatments on the Antioxidant Properties of Wheat Bran

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3.1 Abstract

The bran fraction of wheat grain is known to contain significant quantities of bioactive components. This study evaluated the potential of solid-state enzyme treatments to release insoluble bound antioxidants such as phenolic acids from wheat bran, thereby improving its extractable and potentially bioaccessible antioxidant properties including scavenging capacities against peroxyl (ORAC), ABTS cation, DPPH and hydroxyl radicals, total phenolic contents, and phenolic acid compositions. Investigated enzyme preparations included Viscozyme L, Pectinex 3XL, Ultraflo L, Flavourzyme 500L, Celluclast 1.5L, and porcine liver esterase. Results showed significant dose dependent increases in extractable antioxidant properties for some enzyme preparations, and found Ultraflo L to be the most efficient enzyme, able to convert as much as 50% of the insoluble bound ferulic acid in wheat bran to soluble free form. The effect of moisture content on these solid-state enzyme reactions was also evaluated, and found to be dependent on enzyme concentration. These data suggest that solid-state enzyme treatments of wheat bran may be a commercially viable post-harvest procedure for improving the bioaccessibility of wheat antioxidants.

3.2 Introduction

Increasing evidence supporting the role of bioactive food components in preventing and managing health conditions has promoted research to improve the production of bioactives in food crops and their bioavailability in humans. The consumption of foods rich in antioxidants, one type of bioactive food component, has been linked through epidemiological studies to reduced incidences of chronic diseases such as cancer, heart disease, and diabetes (*1, 2*). Antioxidants are believed to prevent chronic diseases by preventing oxidative damage to important biomolecules such as DNA, membrane lipids, and proteins, through multiple mechanisms (*1-4)*. These mechanisms may include, but are not limited to quenching free radicals, chelating transition metals, or stimulating antioxidative enzyme systems (*1-4*).

Wheat is an important dietary staple and has been found in numerous studies to contain significant antioxidant properties *in-vitro* such as chelating activities against Fe^{2+} and Cu^{2+}, inhibition of low-density lipoprotein and DNA oxidation, and scavenging activities against peroxyl, hydroxyl, DPPH, ABTS cation, and superoxide anion radicals (*5-25*). Phenolic acids present in wheat are thought to significantly contribute to these antioxidant properties and the health benefits of whole grain consumption observed in numerous epidemiological studies (*26, 27*). Significant levels of phenolic acids, predominately ferulic acid, have been detected in both hard and soft wheat grains (*6, 11, 13, 16, 21, 28*) and are found mostly concentrated in the bran fraction (*12, 29-31*). Although some of these phenolic acids exist in free or soluble conjugated forms, the majority are in an insoluble bound form, esterified to

plant cell wall material (*6, 10, 16, 24, 32-34*).

The proposed health benefits of phenolic acids and their significant levels in wheat bran have made their bioavailability in humans of recent interest (*32*). Since only free and some conjugated phenolic acids are thought to be available for absorption (bioaccessible) in the human small or large intestines, human absorption of the predominately bound phenolic acids in wheat bran has been shown to be minimal (*35*). It is widely accepted that free phenolic acids are absorbed in the small intestine while bound phenolics could be minimally absorbed in the colon after hydrolysis from the polysaccharide matrix by colonic microflora (*32, 35*). Given this, the release of bound wheat bran phenolic acids in wheat bran prior to consumption through post-harvesting procedures could be a strategy to improve their bioaccessibility in humans.

The enzymatic hydrolysis of bound phenolic acids from cell wall materials including wheat bran has been previously investigated to produce a natural source of ferulic acid for flavor or pharmaceutical applications (*34*). The enzymatic approach has been shown to be effective using xylanases, β-gluconases, and cellulases to break up wheat bran cell wall material combined with enzymes specifically to hydrolyze the ester linked phenolic acids such as cinnamoyl or feruloyl esterases (*34, 36-43*). These studies, however, conducted the enzyme reactions in aqueous systems, which may prove impractical for commercial scale post-harvest treatments for food ingredients. Solid-state fermentation, however, is widely used commercially to produce foods such as tempeh, miso, and soy sauce (*44*), and to increase the phenolic potential of soybean powder (*45*). The biochemical nature of fermentation is enzyme-catalyzed

reactions. Solid state enzymatic procedures have been developed and used to improve the physiochemical and functional properties of psyllium (*9*), to hydrolyze chestnut starch (*46*), and to enhance the release of phenolics from cranberry pomace (47). Solid-state enzymatic reaction systems are practical for food ingredient production because they require no expensive equipment, are environmentally friendly, and require little post-reaction processing to recover products.

Post-harvest solid-state enzymatic procedures may be utilized to release insoluble bound phenolic acids from wheat bran and thereby improve their bioaccessibility and potential bioavailability. The objective of this study was therefore to evaluate five selected commercially available food-grade enzyme preparations and one purified enzyme for their potential to improve the extractable free phenolic contents and antioxidant properties of wheat bran through solid-state enzymatic reactions. In addition, this study examined the effects of enzyme to substrate ratios and reaction moisture contents on these extractable phenolic contents and antioxidant properties of wheat bran. No study to date has investigated this opportunity.

3.3 Materials and Methods

Chemicals and Reagents. 2,2'-bipyridyl, 2,2-diphenyl-1-picryhydrazyl radical ($DPPH^{\bullet}$), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), fluorescein (FL), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), and $FeCl_3$ were purchased from Sigma-Aldrich (St. Louis, MO). 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). Viscozyme L, Pectinex 3XL, Flavorzyme 500L, and Celluclast 1.5L, all enzyme preparation products produced by Novozymes Corp. (Bagsvaerd, Denmark), were purchased from Simga-Aldrich with product numbers of V2010, P2736, P6100, and C2730. Porcine liver esterase was purchased from Sigma-Aldrich (E2884). Ultraflo L enzyme preparation was a gift from Novozymes North America (Franklinton, NC). Details of all enzyme properties are listed in **Table 3.1**. All other chemicals and solvents were of the highest commercial grade and used without further purification.

Hard Winter Wheat Bran Samples. Bran from Akron and Jagalene wheat varieties, both commonly produced red winter wheat varieties, were provided by Dr. Scott Haley in the Department of Soil and Crop Science, Colorado State University, Fort Collins, CO 80523. Both varieties were grown in Fort Collins, Colorado during the 2004 growing season under agronomic practices considered typical for wheat production in eastern Colorado. Harvested grain samples from each location were cleaned using seed cleaners to remove all non-grain debris present and stored under ambient conditions. Grain samples were ground and separated into flour and bran using a Brabender Quadromat Junior experimental mill.

Table 3.1. Summary of enzyme preparation characteristics

Enzyme Preparation Name	Enzyme Class	Declared Major Activity	Reported Side Activities	Source
Viscozyme L	β-glucanase (endo-1,3(4)-)	100 FBG/g	Xylanase†, Hemicellulase†, Cellulase,† Feruloyl esterase[b]	*Aspergillus aculeatus*
Porcine Liver Esterase	Carboxylic esterase	1667 Esterase units/mL		Porcine Liver
Pectinex 3XL	Polygalacturonase	3000 PECTU/mL	Pectinesterase†, Hemicellulase†, Cellulase,† Xylanase[c]	*Aspergillus aculeatus* and *Aspergillus niger*
Ultraflo L	β-glucanase (endo-1,3(4)-)	45 FBG/g	Arabanase†, Cellulase†, Pentosanase†, Xylanase,† Feruloyl esterase[a]	*Humicola insolens*
Flavourzyme 500L	Aminopeptidase	500 LAPU/g		*Aspergillus oryzae*
Celluclast 1.5L	Cellulase	700 EGU/g		*Trichoderma reesei*

† = identified by manufacturer

[a] = see reference (*42, 60*)

[b] = see reference (*59, 60*)

[c] = see reference (*59*)

EGU stands for endo glucanase units; LAPU stands for leucine aminopeptidase units; FBG stands for fungal β-glucanase units; PECTU stands for pectinase units.

Solid-state Enzymatic Reactions. Bran samples were ground to 40-mesh using a micro-mill manufactured by Bel Art Products (Pequannock, NJ) and tempered to a moisture content of 10%. The individual enzyme preparations were mixed in wheat bran to initiate the reaction, and the reaction was carried out at ambient temperature in the dark for 72 hours. Enzyme doses were 0 (control), 2.26, 4.52, 9.04, 18.04, and 221.6 U/g wheat bran (on dry weight basis) for each tested enzyme preparation with an initial treatment moisture content of 35% in all ground bran samples. The possible effects of moisture content on solid-state enzymatic reactions were examined using Viscozyme L at initial treatment moisture contents of 30 and 43% in the bran with enzyme doses of 0, 2.26, 4.52, 9.04, 18.09, and 33.91 U/g wheat bran (on dry weight basis). See **Table 3.1** for enzyme characteristics. Enzymes were inactivated by heating using a microwave oven, and re-ground to 40-mesh.

Sample Extraction Procedure. One gram samples of 40-mesh enzyme treated bran were extracted with 10 mL 100% ethanol for 18 hours under nitrogen in the dark at ambient temperatures. The ethanol extracts were used for $ABTS^{\bullet+}$ scavenging ability, oxygen radical absorbing capacity (ORAC), and $DPPH^{\bullet}$ scavenging activity assays. Known volumes of 100% ethanol extracts were dried under nitrogen, and the solid residue was quantitatively re-dissolved in DMSO for total phenolic contents (TPC) assay or acetone for the hydroxyl radical scavenging capacity assay (HOSC). Extracts were stored under nitrogen in the dark at ambient temperatures until further analysis.

Oxygen Radical Absorbing Capacity (ORAC) Assay. ORAC assay was conducted with fluorescein (FL) as the fluorescent probe using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland) according to a previous laboratory protocol (*16*) with 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 antioxidant extract, standard, or 100% ethanol for blanks, and 25 μL of 0.36 M AAPH. FL and antioxidant extracts were mixed in 96 well plate and pre-heated in plate reader for 20 minutes at 37ºC after which the AAPH solution was added to initiate the antioxidant-radical reactions. The fluorescence of the assay mixture was recorded every minute for 80 minutes at 37ºC. Excitation and emission wavelengths were 485 nm and 535 nm respectively. Results were expressed as μmoles of trolox equivalents (TE) per g wheat bran on a dry weight basis.

Radical Cation ABTS$^{\bullet+}$ Scavenging Capacity. The free radical scavenging capacity of the 100% ethanol extracts was evaluated against ABTS$^{\bullet+}$ generated according to a previously reported protocol (*11, 48*). Fifty μL of the bran extracts were diluted to 500 μL with 100% ethanol to create working sample solutions. ABTS cation radicals were generated by oxidizing a 5 mM aqueous solution of ABTS with manganese dioxide for 30 min at ambient temperature. The final reaction mixture contained 80 μL of working sample solution or 100% ethanol for control, and 1.0 mL ABTS$^{\bullet+}$ solution with an absorbance of 0.7 at 734 nm. The absorbance at 734 nm was measured after a reaction time of 1 minute. Trolox equivalents (TE) were

calculated using a standard curve prepared with trolox and expressed in μmoles TE per gram of wheat bran on a dry weight basis.

Radical DPPH Scavenging Capacity. The $DPPH^{\bullet}$ scavenging capacity of the bran antioxidant extracts was determined following a previously reported procedure with modifications (*49*). Briefly, 100 μL of antioxidant extract or ethanol for blank was added to 100 μL of freshly prepared $DPPH^{\bullet}$ solution to initiate antioxidant-radical reaction. The absorbance of the reaction mixture was measured at 515 nm at 40 minutes of reaction. The initial concentration was of $DPPH^{\bullet}$ was 100 μM for all reaction mixtures. $DPPH^{\bullet}$ radical scavenging capacity was expressed as the percent of $DPPH^{\bullet}$ scavenged in 40 min under the experimental conditions.

Hydroxyl Radical Scavenging Capacity (HOSC) Assay. HOSC assay was conducted with acetone solutions according to a previously published protocol (*23*) using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). Reaction mixtures consisted of 170 μL of 9.28×10^{-8} M FL prepared in 75 mM sodium phosphate buffer, 30 μL of standard or sample or blank, 40 μL of 0.1990 M H_2O_2, and 60 μL of 3.43 mM $FeCl_3$. Fluorescence was measured every minute for 3 hours with an excitation wavelength of 485 nm and emission wavelength of 535 nm. Trolox prepared in acetone at concentrations of 20, 40, 60, 80, and 100 μM were used to prepare the standard curve for HOSC quantification. HOSC results were expressed as micromoles trolox equivalents (TE) per gram of wheat bran on a dry weight basis.

Total Phenolic Contents. The DMSO extracts were analyzed for total

phenolic contents using the Folin-Ciocalteu reagent according to a previously reported procedure (*7, 16*). Folin-Ciocalteu reagent was prepared by refluxing 85% phosphoric acid, sodium molybdate, sodium tungstate, and concentrated hydrochloric acid for 10 h, reacting with lithium sulfate, then oxidizing with bromine followed by filtration. The final reaction mixture contained 50 μL of antioxidant extracts in DMSO, 250 μL freshly prepared Folin-Ciocalteu reagent, 750 μL 20% sodium carbonate, and 3 mL of ultra-pure water. Absorbance at 765 nm was read after a reaction time of 2 h at ambient temperature. Total phenolic contents calculated using gallic acid as a standard.

Phenolic Acid Composition. Treated bran samples were analyzed for their soluble free, soluble conjugated, insoluble bound, and total phenolic acid compositions using a previously reported procedure (*16*). Acetone/methanol/water (7/7/6, v/v/v) was used to extract the soluble free and the soluble conjugated phenolic acids, while the insoluble bound phenolic acids remained in the resulting solid residue. The free and conjugated phenolic acids in the acetone/methanol/water solution were separated based on their solubility in ethyl acetate/ethyl ether (1:1, v/v) under acidic condition (pH = 2). Soluble conjugated phenolic acids were also hydrolyzed using NaOH, and re-extracted in ethyl acetate/ethyl ether (1:1, v/v) after the reaction pH was brought to pH 2. The solid residue with insoluble bound phenolic acids was hydrolyzed with NaOH, and the supernatant was re-extracted with ethyl acetate/ethyl ether (1:1, v/v) after pH was adjusted to about pH 2. The concentration of NaOH in the hydrolysis reaction mixtures was 2 M. After

evaporation of ethyl acetate and ethyl ether, each phenolic acid extract was quantitatively re-dissolved in MeOH and analyzed by HPLC using a Phenomenex C18 column (250 mm × 4.6 mm) according to an established protocol (*11*). 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). Solvent gradient was programmed from 10 to 100% B in 42 min with a flow rate of 1.0 mL/min (*11, 16*). Identification of phenolic acids was accomplished by comparing the retention time of peaks in the samples to that of the standards under the same HPLC conditions. Quantification of each phenolic acid was determined using external standards and total area under each peak.

Moisture Content. The moisture content of bran samples before and after the solid-state enzymatic reactions were determined using an oven following the AACC method 44-16 (*50*).

Statistical analysis. Data were reported as mean ± SD for triplicate determinations. ANOVA and Tukey's tests were performed (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL) to identify differences among means. Statistical significance was declared at $P \leq 0.05$.

3.4 Results and Discussion

Wheat is an important dietary staple with over 600 million metric tons estimated to be produced world-wide in 2006 (*51*). The important antioxidants in wheat grain, phenolic acids, are concentrated in the bran fraction and are thought to

contribute to the disease prevention properties attributed to wheat bran or whole grain consumption in epidemiological studies (*26, 52, 53*). The majority of the phenolic acids present in wheat, however, are only minimally available for absorption (bioaccessible) because they are bound to wheat bran cell wall materials in the aleurone and pericarp layers through ester linkages (*52, 32, 35*).

The polysaccharide composition of wheat bran aleurone and pericarp cell walls includes mostly arabinoxylans with some β-glucans and cellulose (*33, 34*). Phenolic acids are primarily esterified to the C-5 hydroxyl group of α-L-arabinofuranosyl substituents which are linked to C-2 or C-3 on the xylopyranosyl backbone. (*34, 36*). Previous studies on the enzymatic breakdown of cell wall materials for release of phenolic acids have found the most effective enzyme mixtures to include xylanses, α-L-arabinofuranosidases, acetyl xylan esterases, α-glucoronidases, and ferulic and p-coumaric esterses (*39*). These studies have used either mixtures of purified enzymes and isolated enzymes from microorganisms, or commercial mixed enzyme preparations such as Ultraflo-L, Viscozyme-L, Celluclast 1.5L, Termamyl and Lallzyme in submerged aqueous reaction systems. (*37, 42, 54*).

The present study utilized a solid-state enzymatic reaction system to release the phenolic acids present in wheat bran. Solid-state reaction systems are used in the production of many food ingredients and have been researched for the enzymatic release of phenolics from materials such as soybean powders, pineapple residue, cranberry pomace, and black current juice residue (*45, 47, 55, 56*). Solid-state enzymatic reaction systems are more commercially practical for food ingredient

modification and production than the aqueous phase reaction systems because there is no additional step needed after inactivation of enzyme(s) to remove water or isolate the final products. In addition, the solid-state enzymatic procedure generates no waste and requires no special equipment.

Effects of Different Enzyme Preparations on the Antioxidant Properties of Hard Wheat Bran. Five commercial food-grade enzyme mixture preparations including (Viscozyme L, Pectinex 3XL, Ultraflo L, Flavourzyme 500L, and Celluclast 1.5L), and Porcine Liver Esterase (a purified non-food grade enzyme) were used to treat Akron and Jagalene hard wheat bran at doses of 0-18 units per gram under the solid-state reaction conditions. All enzymes were additionally tested at a dose of 221 U/g, except Viscozyme L and Ultraflo L preparations which were not concentrated enough to allow this high dosage. To increase the scope of results for this study, bran samples of two hard wheat varieties were included. Statistical analysis for each antioxidant assay was performed between treatments within each variety to determine any significant differences. Individual enzyme preparation differed in their effects on bran antioxidant properties including oxygen radical absorbing capacity (ORAC), $ABTS^{\bullet+}$ scavenging capacity, $DPPH^{\bullet}$ scavenging capacity, and total phenolic contents.

Oxygen Radical Absorbing Capacities (ORAC) —ORAC measures peroxyl radical scavenging capacity expressed as μmoles trolox per gram wheat bran on a dry weigh basis. Solid-state enzymatic treatment significant increased ORAC values of both Akron and Jagalene wheat bran samples (**Figures 3.1a-b**). The largest increases

in ORAC value versus the control were 4.3 fold for Akron wheat bran treated with 221 U/g Celluclast 1.5L, and a 3.5 fold increase for Jagalene treated with 9 U/g Ultraflo-L. The most efficient enzyme, showing the greatest percent increase per unit of enzyme activity, was Ultraflo L for bran samples of both wheat varieties. Other enzyme preparations showed ORAC value increases of 1.8, 2.4, 4.0 and 3.6 fold for Akron wheat bran treated with Porcine Esterase, Pectinex 3XL, Ultraflo L and Flavourzyme 500L, respectively, while treatments of Jagalene bran showed increases of 1.4, 1.8, 2.6, 2.7, and 2.3 fold for Viscozyme L, Porcine Esterase, Pectinex 3XL, Flavourzyme 500L, and Celluclast 1.5L respectively. Results for all treatments indicated that ORAC value increases for enzyme treatments were dose dependent, except for Pectinex 3XL treatments.

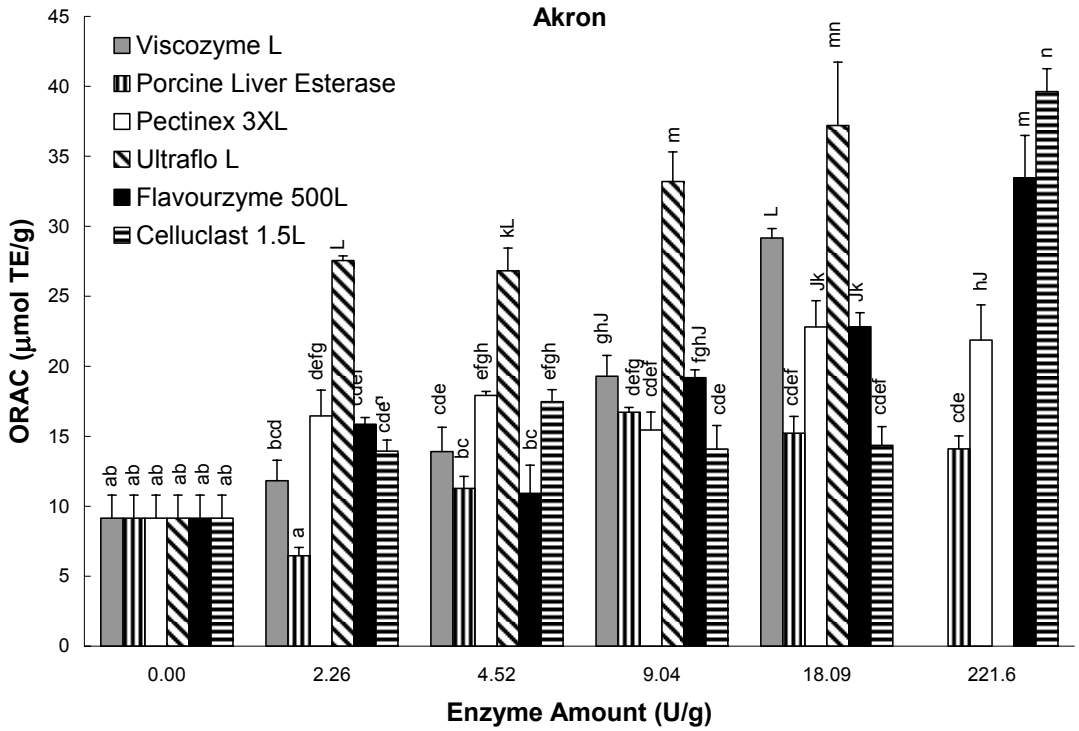

Figure 3.1a. Effects of different enzyme treatments on the oxygen radical absorbing capacities (ORAC) for Akron wheat bran. Enzyme treatments of bran carried out at moisture content of 20% for all treatments. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See Table 3.1 for enzyme characteristics. Results expressed as µmoles trolox equivalents per gram of wheat bran on a dry weight basis. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

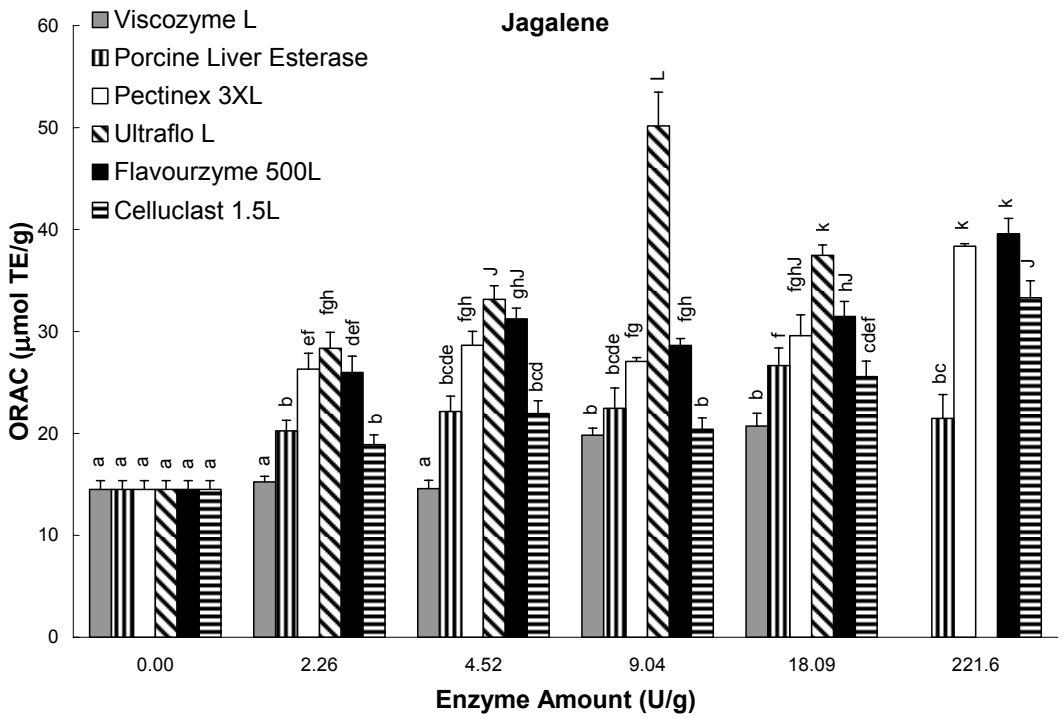

Figure 3.1b. Effects of different enzyme treatments on the oxygen radical absorbing capacities (ORAC) for Jagalene wheat bran. Enzyme treatments of bran carried out at moisture content of 20% for all treatments. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See Table 3.1 for enzyme characteristics. Results expressed as µmoles trolox equivalents per gram of wheat bran on a dry weight basis. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

$ABTS^{\bullet+}$ Scavenging Capacities

$ABTS^{\bullet+}$ scavenging capacities of enzyme-treated bran samples were measured and expressed as µmoles trolox per gram bran on a dry weigh basis. Some enzyme treatments resulted in significant increases in $ABTS^{\bullet+}$ scavenging capacity of the bran (**Figure 3.2a-b**). For both Akron and Jagalene wheat bran samples Ultraflo L was the most efficient enzyme preparation showing the greatest increases of $ABTS^{\bullet+}$ scavenging capacity on a per unit of enzyme activity basis, and also showed the

highest increases (1.7 fold) at 18 U/g dosage level. Flavourzyme 500L treatments showed comparable increases, but at the high enzyme level of 221 U/g. For both bran samples, Porcine Esterase treatments showed some significant decreases in $ABTS^{\bullet+}$ scavenging capacity versus control at low concentrations, and no significant differences from control at other levels. Other enzymes for both varieties showed some small significant increases, but only at higher enzyme levels. Results in **Figure 3.2** also indicated that $ABTS^{\bullet+}$ scavenging capacity increases for enzyme treatments were dose dependent except for Celluclast 1.5L treatments.

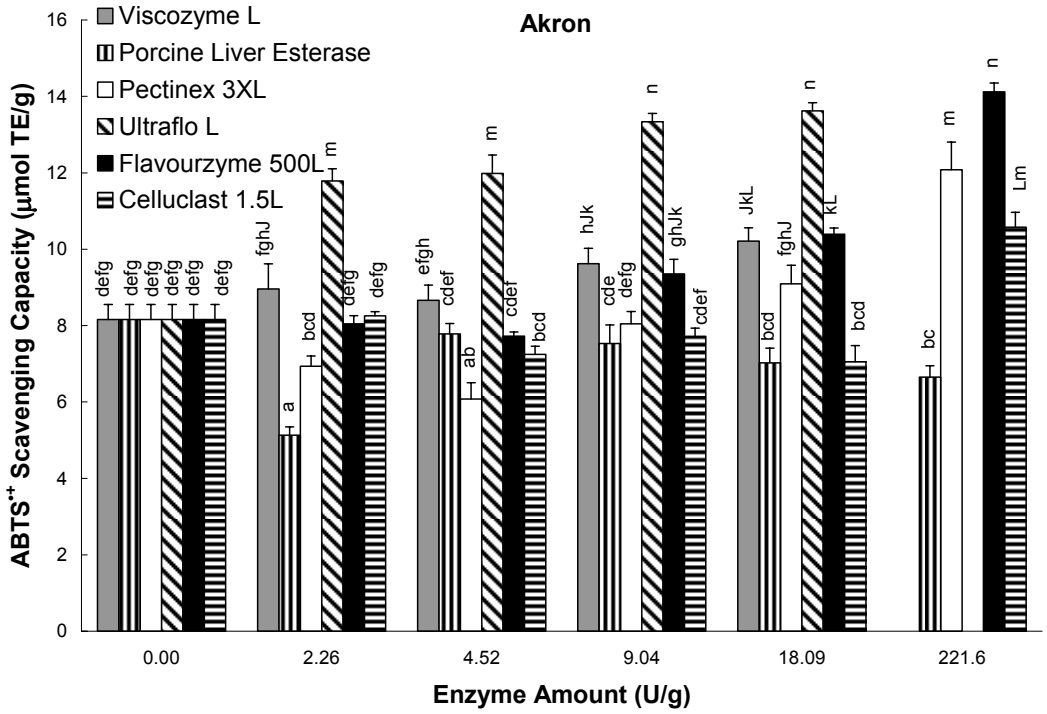

Figure 3.2a. Effects of different enzyme treatments on the $ABTS^{\bullet+}$ scavenging capacities for Akron wheat bran. Enzyme treatments of bran carried out at moisture content of 20% for all treatments. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See Table 3.1 for enzyme characteristics. Results expressed as µmoles trolox equivalents per gram of wheat bran on a dry weight basis. All tests were conducted in duplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

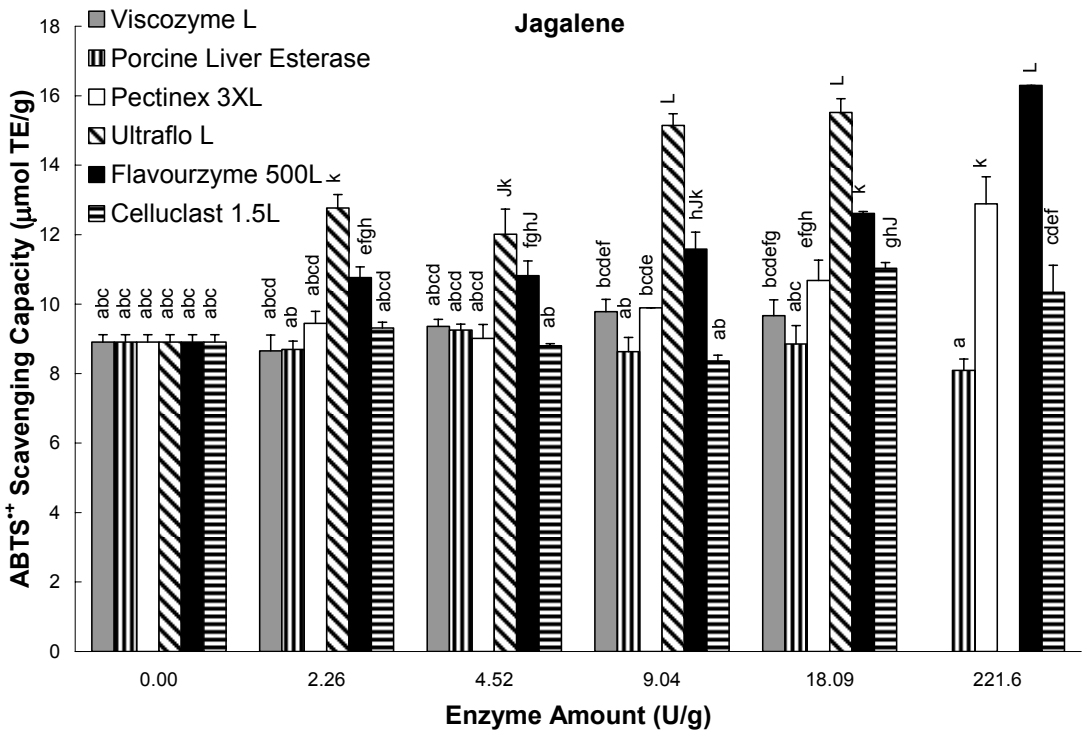

Figure 3.2b. Effects of different enzyme treatments on the ABTS$^{\bullet+}$ scavenging capacities for Jagalene wheat bran. Enzyme treatments of bran carried out at moisture content of 20% for all treatments. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See Table 3.1 for enzyme characteristics. Results expressed as µmoles trolox equivalents per gram of wheat bran on a dry weight basis. All tests were conducted in duplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

DPPH$^{\bullet}$ Scavenging Capacities

DPPH$^{\bullet}$ scavenging capacities of the enzyme-treated wheat bran samples are shown in **Figure 3.3a-b** expressed as percent DPPH$^{\bullet}$ scavenged. For treatments of Akron bran, Ultraflo L was the most efficient enzyme preparation and had the highest increases of 1.8 fold at 18 U/g dosage, while Flavourzyme 500L treatments showed comparable increases, but at higher enzyme doses. For Jagalene treatments, both Ultraflo L and Flavourzyme 500L showed similar efficiencies with the greatest

percent increases of 1.8 fold at 18 and 221 U/g respectively. Porcine Esterase treatments for both varieties showed no significant increases versus the control, and for Akron at 2 U/g showed a significant decrease in DPPH$^{\bullet}$ scavenging capacity. Other enzyme treatments for both varieties showed some small increases in DPPH$^{\bullet}$ scavenging capacities at higher enzyme levels. Results for all treatments indicated that DPPH$^{\bullet}$ scavenging capacity increases for enzyme treatments were dose dependent except for Porcine Esterase treatments for Jagalene bran. In addition, Cellulcast 1.5L treatment decreased DPPH$^{\bullet}$ scavenging capacity in Akron bran, but dose-dependently increased DPPH$^{\bullet}$ scavenging capacity in Jagalene bran (**Figure 3.3**), suggesting that the bran sample may respond to a selected enzyme differently.

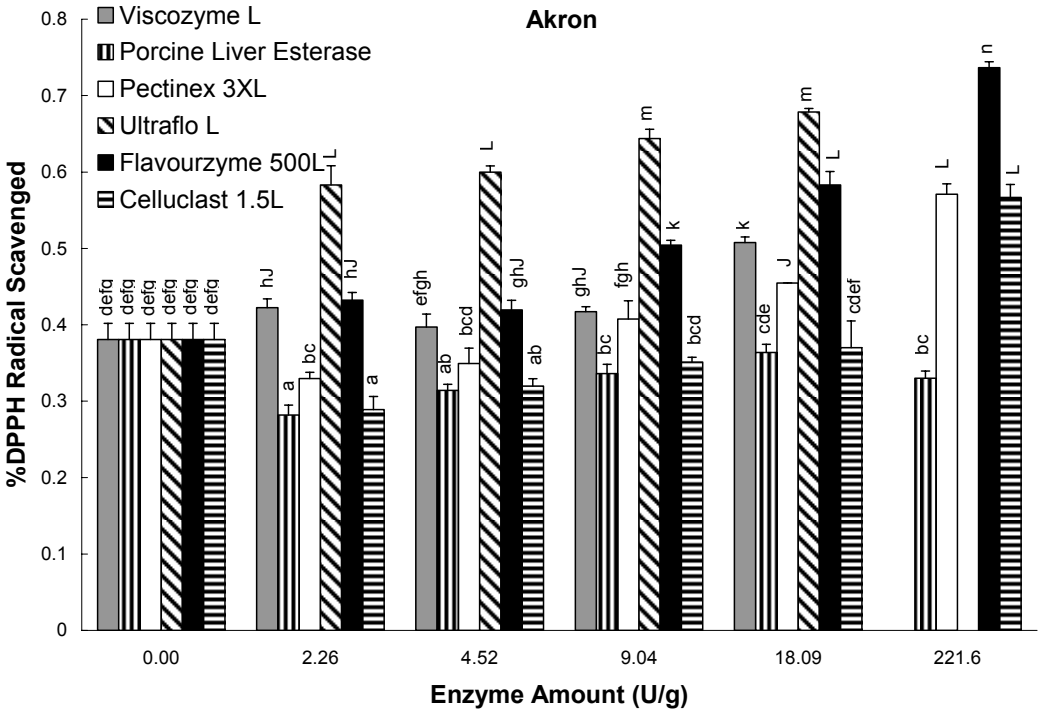

Figure 3.3a. Effects of different enzyme treatments on the DPPH• scavenging capacities for Akron wheat bran. Enzyme treatments of bran carried out at moisture content of 20% for all treatments. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See Table 3.1 for enzyme characteristics. Results expressed percent DPPH• scavenged after 40 minutes reaction time. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

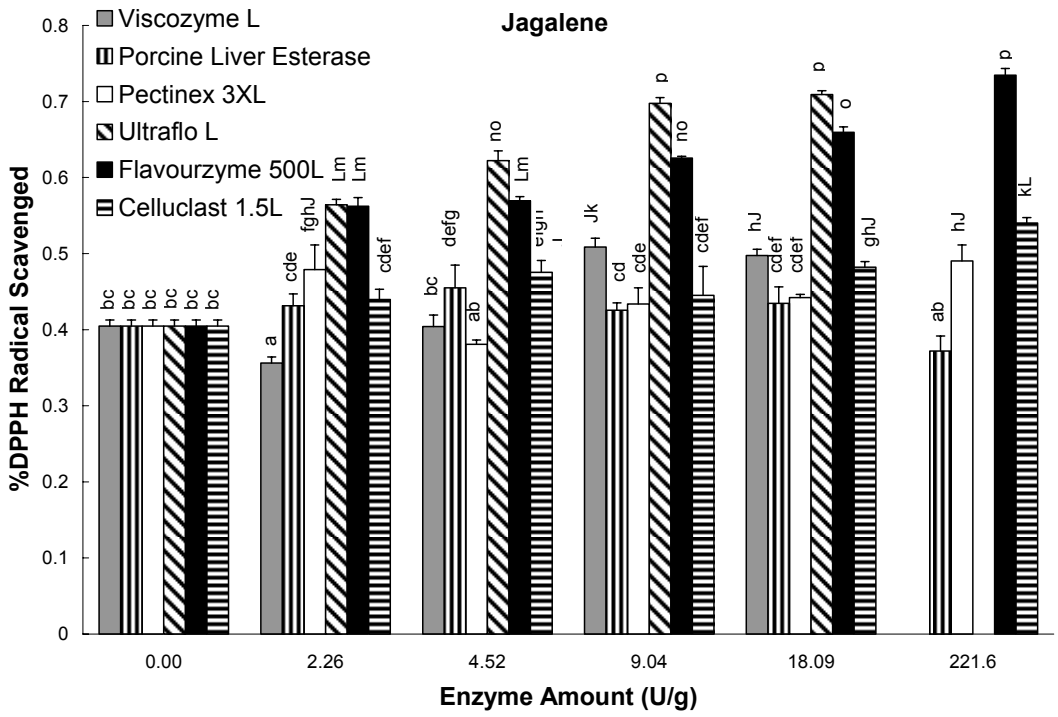

Figure 3.3b. Effects of different enzyme treatments on the DPPH• scavenging capacities for Akron wheat bran. Enzyme treatments of bran carried out at moisture content of 20% for all treatments. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See Table 3.1 for enzyme characteristics. Results expressed percent DPPH• scavenged after 40 minutes reaction time. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

Hydroxyl Radical Scavenging Capacity (HOSC)

The hydroxyl radical scavenging capacity of enzyme-treated wheat bran samples are shown in **Figure 3.4a-b** with results expressed as μmoles trolox equivalents per gram wheat bran on a dry weigh basis. Results showed some significant increases in HOSC values for both varieties of wheat bran, dependent on enzyme preparation and dose. The largest increases in HOSC versus control were Ultraflo L treatments for both varieties of wheat bran, showing 4.5 and 2.76 fold for

Arkon at 9 U/g and Jagalene at 18 U/g. Ultraflo L was also observed to be the most efficient enzyme preparation tested, with the greatest increase in hydroxyl radical scavenging capacity per unit of enzyme. Other enzyme preparations showed maximum increases in HOSC for Akron of 2.2, 1.9, 1.5, 4.0, and 3.5 fold for Viscozyme L, Porcine Esterase, Pectinex 3XL, Flavourzyme 500L, and Celluclast 1.5L. Treatments of Jagalene bran showed increases of 1.5, 1.2, 2.1, 2.0, and 2.5 fold for Viscozyme L, Porcine Esterase, Pectinex 3XL, Flavourzyme 500L, and Celluclast 1.5L respectively.

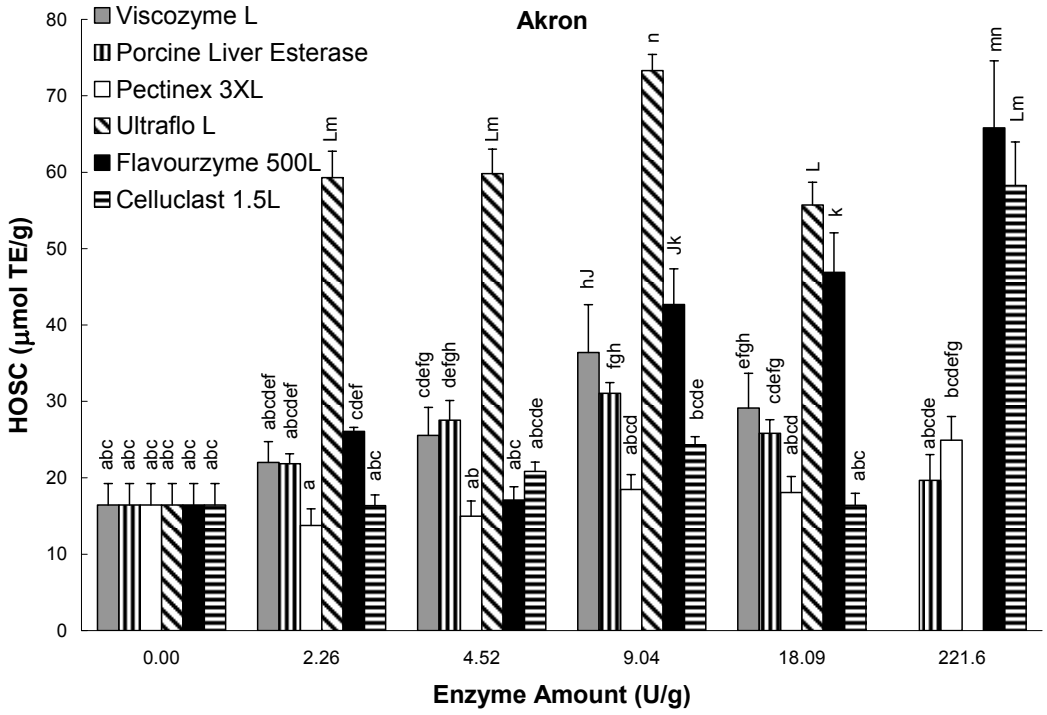

Figure 3.4a. Effects of different enzyme treatments on the hydroxyl radical scavenging capacities (HOSC) for Akron wheat bran. Enzyme treatments of bran carried out at moisture content of 20% for all treatments. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activity for Viscozyme L. See Table 3.1 for enzyme characteristics. Results expressed as µmoles trolox equivalents per gram of wheat bran on a dry weight basis. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

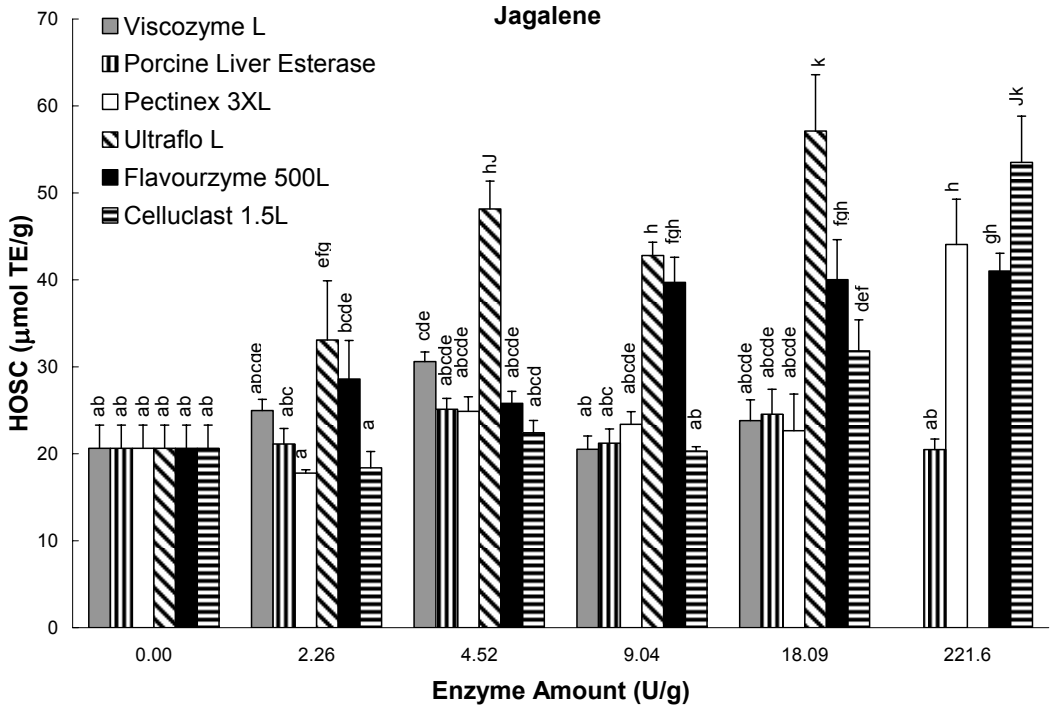

Figure 3.4b. Effects of different enzyme treatments on the hydroxyl radical scavenging capacities (HOSC) for Jagalene wheat bran. Enzyme treatments of bran carried out at moisture content of 20% for all treatments. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activity for Viscozyme L. See Table 3.1 for enzyme characteristics. Results expressed as µmoles trolox equivalents per gram of wheat bran on a dry weight basis. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

Total Phenolic Contents

Total phenolic contents (TPC) were measured for enzyme treated bran sample using the Folin-Ciocalteu reagent, and expressed as mg gallic acid equivalents per gram bran on a dry weight basis. Individual enzyme preparation differed in their capacity to enhance the releasable levels of total phenolic compounds from Akron and Jagalene bran samples (**Figure 3.5a-b**). For both Akron and Jagalene wheat bran samples Ultraflo L was the most efficient enzyme preparation showing the greatest

capacity to increase releasable TPC per unit of enzyme activity, and showed maximum increases of 4.5 and 3.3 fold at the enzyme concentration of 9 U/g respectively. Regardless of wheat variety, increasing enzyme concentration in the solid-enzymatic reaction mixture from 0 to 9 U/g increased the extractable amount of TPC, but further increase of Ultraflo L concentration from 9 to 18 U/g did not increase the level of releasable TPC under the experimental conditions. Flavourzyme 500L treatments also dose-dependently increased the amount of extractable TPC in both Akron and Jagalene wheat bran samples under the experimental conditions (**Figure 3.5**). Flavourzyme 500L treatments at a level of 221 U/g resulted in the greatest increases of TPC, with 5.6 and 5.1 fold increases for Akron and Jagalene respectively. Porcine Liver Esterase treatments were not able to improve the TPC release from either Akron or Jagalene wheat bran samples, under the sample testing conditions, and lead to decreased TPC for Akron bran at 2 and 4 U/g (**Figure 3.5**). Viscozyme L, Pectinex 3XL, and Celluclast 1.5L were able to slightly and dose-dependently enhance the extractable TPC levels (**Figure 3.5**).

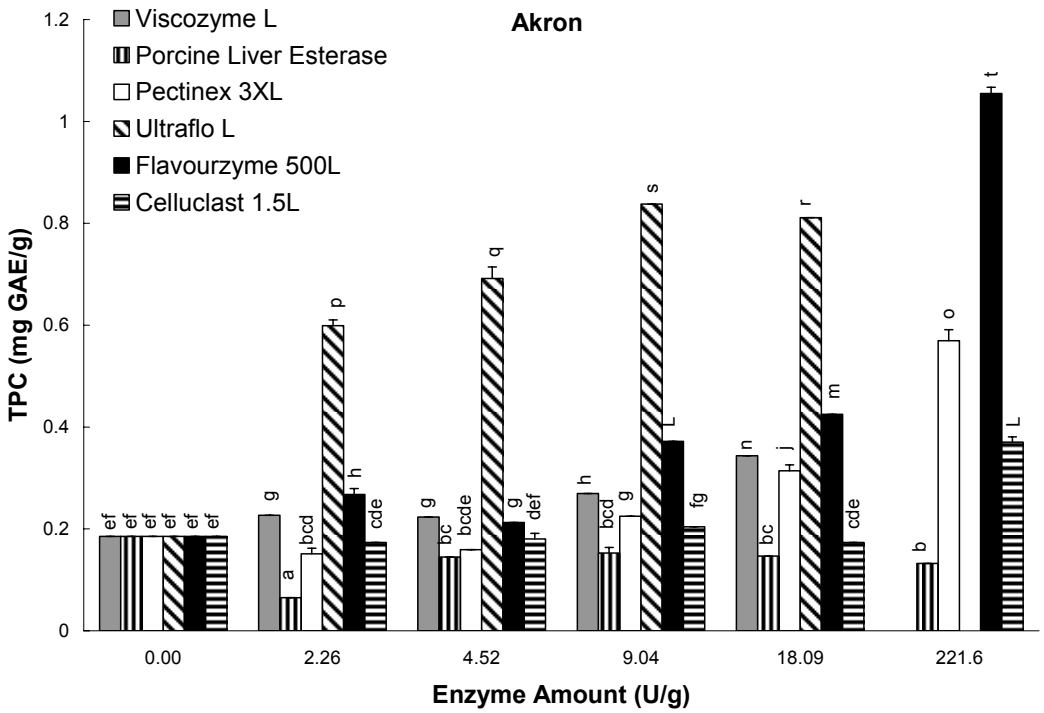

Figure 3.5a. Effects of different enzyme treatments on the total phenolic content (TPC) for Akron wheat bran. Enzyme treatments of bran carried out at moisture content of 20% for all treatments. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See Table 3.1 for enzyme characteristics. Results expressed mg gallic acid equivalents (GAE) per gram of wheat bran on a dry weight basis. All tests were conducted in duplicated, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

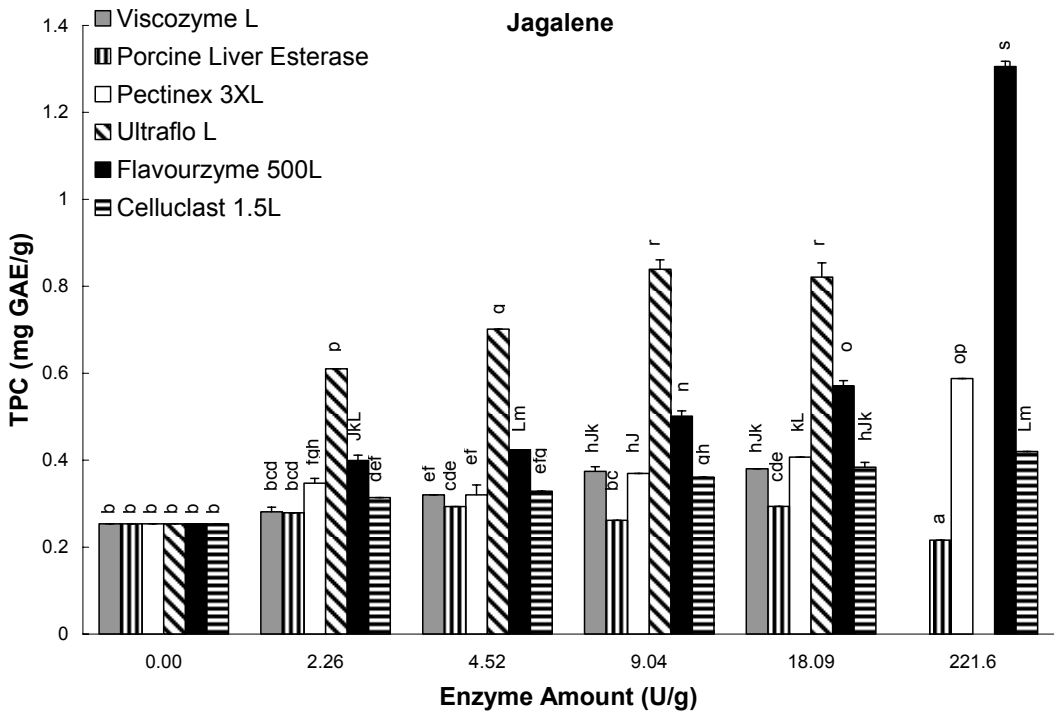

Figure 3.5b. Effects of different enzyme treatments on the total phenolic content (TPC) for Jagalene wheat bran. Enzyme treatments of bran carried out at moisture content of 20% for all treatments. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See Table 3.1 for enzyme characteristics. Results expressed mg gallic acid equivalents (GAE) per gram of wheat bran on a dry weight basis. All tests were conducted in duplicated, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

The results of these antioxidant activity evaluations show for the first time that solid-state enzyme treatments may increase the *in-vitro* antioxidant activities of wheat bran. These increases in antioxidant activity are likely a result of the enzymatic release of the insoluble bound phenolic acids, the predominant form of antioxidant compounds in wheat. These results also suggest that Ultraflo-L may be more efficient, on per enzyme activity unit basis, than other tested enzyme preparations for enhancing the extractable antioxidant components and thereby increasing *in-vitro* antioxidant activities of wheat bran. Ultraflo L is considered a well balanced mixture

of cell wall degrading enzymes including beta-glucanase as the primary enzyme activity and other reported side activities including arabinase, cellulase, pentosanase, xylanase to degrade the xylan backbone, and importantly significant feruloyl esterase activity to release bound ferulic acid, the predominant phenolic acid in wheat (*42*). Interestingly, Flavourzyme 500L, a peptidase, was able to significantly increase the releasability of bran antioxidants under the solid-state enzymatic reaction conditions, suggesting that this enzyme preparation may be capable of hydrolyzing ester and glycosidic bonds. The purified esterase (porcine liver esterase) did not significantly increase extractable antioxidant activities at all tested concentrations or antioxidant properties, suggesting that this esterase may not be able to specifically hydrolyze hydroxycinnamate ester bonds in wheat bran due to the possible steric hindrance of the polysaccharide structure and the limited migration of enzyme under the solid-state reaction conditions. Solid-state reaction with Pectinex 3XL, a preparation with primary polygalacturonase activity, might significantly increase the releasable antioxidant properties, but mostly at high concentrations indicating that the reported side activities of the enzyme preparation such as the xylanase and hemicellulase activities (see **Table 3.1**) may be responsible for these increases. Viscozyme L and Celluclast 1.5L are two commonly used cell wall degrading enzyme preparations, Celluclast containing primarily cellulase activity while Viscozyme L has mixed xylanase, hemicellulase, and cellulase activities. Viscozyme L treatment resulted in significant increases in antioxidant activity, but not as effective as Ultraflo L on per enzyme unit basis under the solid-state reaction conditions. This is supported by a study from Bartolome and others (*54*) showing Ultraflo L to be superior to

Viscozyme L and other enzyme preparations for enhancing the release of phenolic acids from barley using aqueous phase reactions. Celluclast 1.5L treatment also resulted in some significant increases in antioxidant activities, but mostly at higher enzyme doses. This result agrees with the fact that the wheat bran cell wall matrix contains mostly arabinoxylans which cannot be hydrolyzed by cellulases, but at high concentrations of Celluclast 1.5L, its side activities may be able to hydrolyze the matrix.

The above results comparing different enzyme preparations suggest that primary enzyme activity in addition to side activities (accessory enzymes) play an important role in determining the ability of an enzyme preparation to increase the extractable antioxidant properties of wheat bran. This is supported by numerous studies which have suggested that wheat bran main-chain (xylan) depolymerizing enzymes work in combination or possible synergy with side-group cleaving accessory enzymes such as arabinases, and ferulic acid esterases to provide the most effective enzyme system for breaking down cell wall material and releasing ferulic acid from wheat bran (*39, 42*). This can be explained in that hydrolysis of the xylan backbone of wheat bran cell wall material enhances the accessibility of ferulic acid esterases and arabinases to their substrates and vice versa, providing a synergistic effect. Other studies have also suggested that the different types of xylanases which have different specificities for xylan hydrolysis dependent on substitution of the backbone, can significantly affect the effectiveness of these enzyme systems for degrading wheat bran cell wall materials (*42*). Lastly, a study by Sørensen and others (*37*) showed that

xylanases present in Ultraflo L and Celluclast 1.5L enzyme preparations may have synergistic interactions in their abilities to hydrolyze wheat bran arabinoxylans, suggesting a possible synergism between these two preparations in releasing phenolic acids release from wheat bran. Together, the results of this and other studies suggest the opportunity for evaluating the synergistic effects of different enzyme combinations in releasing extractable antioxidants from wheat bran in solid-state reaction systems.

Soluble and Insoluble Bound Phenolic Acid Compositions

The effects of solid-state enzymatic treatments on total soluble and insoluble bound phenolic acid compositions in wheat bran were investigated using the Viscozyme L and Ultraflo L enzyme preparations. The total soluble including soluble free and conjugated, and insoluble bound phenolic acid compositions of the selected treatment samples were determined using HPLC and compared to that of a control sample which went through the solid-state reaction procedure without enzyme addition. Results shown in **Figure 3.6a** show the changes in individual phenolic acids for Viscozyme L treatments of Akron wheat bran at 0 (control), 4.5, and 18 U/g enzyme levels. **Figure 3.6b** shows Ultraflo L treatments of Akron wheat bran at 0 (control), 2, and 18 U/g. Significant concentrations of four phenolic acids were detected in treated and control samples including vanillic, syringic, *P*-coumaric, and ferulic acids. All analyzed treatments showed no detectable soluble conjugated phenolic acids. For Viscozyme L treatments, bound phenolic acids decreased dependent on enzyme level with individual ranges of 22-14, 21-13, 38-21, and 1200

to 635 μg/g bran for vanillic, syringic, p-coumaric, and ferulic acids respectively, while free phenolic acids increased dose-dependently with ranges of 21-40, 3.2-11, and 7– 95 μg/g bran for vanillic, syringic, and ferulic acids. Similar dose dependencies and concentration ranges for bound and free phenolic acids in Ultraflo L treatments were observed except for free ferulic acid which had significantly higher concentrations of 433 and 173 μg/g bran at 2 and 18 U/g treatment levels indicating decreased free ferulic concentrations with increased enzyme levels.

Results for these two commercial enzyme preparations to release ferulic and vanillic acids from wheat bran samples indicate that Ultraflo L hydrolyzed more insoluble bound phenolic acids than Viscozyme L. These results agree with the antioxidant property results in **Figures 3.1–3.4** which indicate that Ultraflo L was superior to Viscozyme L in improving the extractable antioxidant properties of both wheat bran samples. The HPLC results were also supported by a previous study (*54*) which found that Ultraflo L was able to release more ferulic and *p*-coumaric acids from barley spent grain than Viscozyme L. In addition, these results were supported by a study conducted by Sørensen and other (*37*) which showed that Ultraflo L was superior to Viscozyme L at hydrolyzing wheat arabinoxylans which are thought to the main polysaccharide matrix to which phenolic acids are bound to in wheat bran.

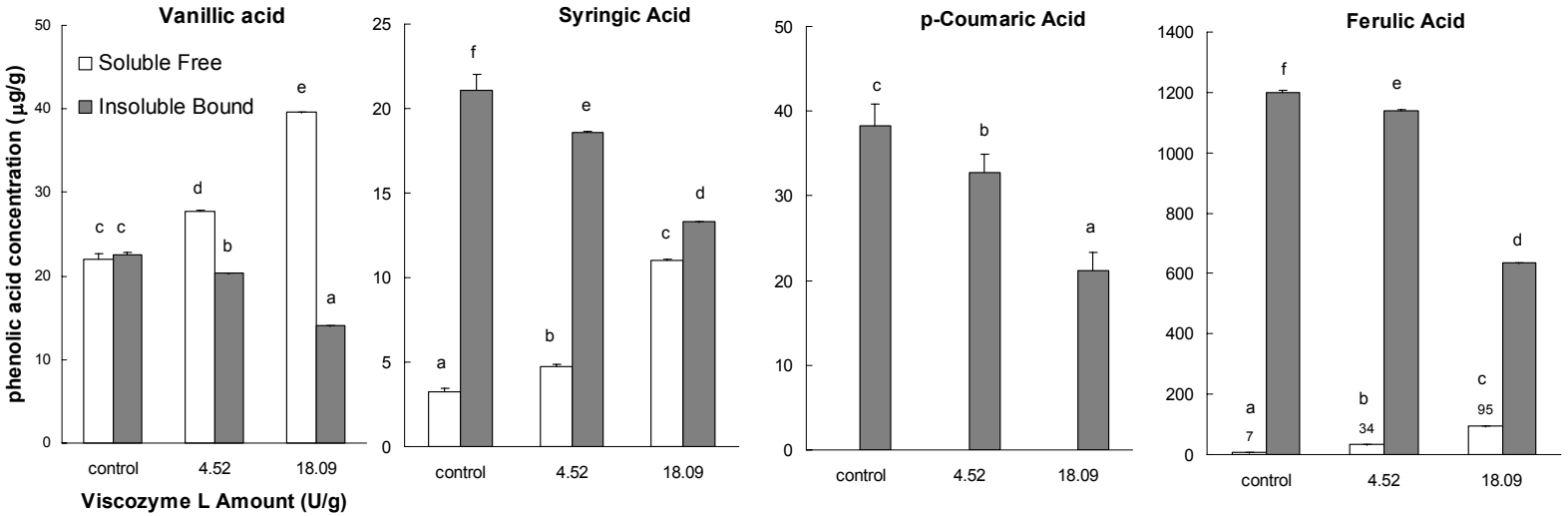

Figure 3.6a. Effects of Viscozyme L treatments on the phenolic acid composition of Akron wheat bran. Enzyme treatments of bran carried out at moisture content of 20% for all treatments. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See Table 3.1 for enzyme characteristics. Results expressed µg individual phenolic acids per gram of wheat bran on a dry weight basis. All tests were conducted in duplicated, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

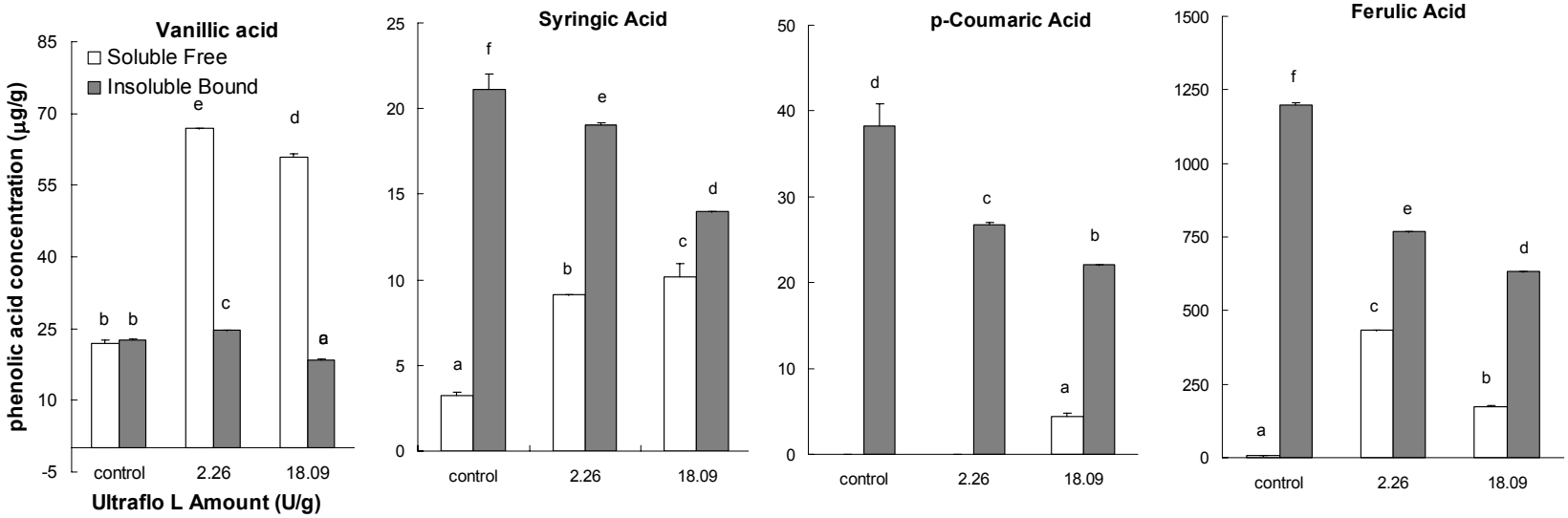

Figure 3.6b. Effects of Ultraflo L treatments on the phenolic acid composition of Akron wheat bran. Enzyme treatments of bran carried out at moisture content of 20% for all treatments. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activities for each enzyme preparation. See Table 3.1 for enzyme characteristics. Results expressed µg individual phenolic acids per gram of wheat bran on a dry weight basis. All tests were conducted in duplicated, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

The percent bound ferulic acid release from wheat bran found in this study using the most effective enzyme preparation, Ultraflo L, ranged from 22 to 50% at the enzyme concentrations evaluated in this study (**Figure 3.6b**). These results while lower than those from a previous study using an aqueous phase enzyme reaction, which showed 90% release using the same enzyme preparation on wheat bran (*42*), demonstrate significant potential for application of these solid-state reactions commercially where aqueous phase reactions are not viable.

In particular, this release of free phenolic acids may have applications in improving the bioaccessibility and therefore bioavailability of wheat bran phenolics. It is well accepted that soluble free phenolic acids are readily bioaccessible in the human small intestine and are a major contributor to the absorbable phenolics present in wheat (*57*). The majority of wheat phenolics, however, are present in an insoluble bound form which are thought to be bioaccessible only in colon where microflora could hydrolyze them to free phenolics followed by further metabolism, or absorption in the colon (*16, 57*). A recent human study by Kern and others (*35*) which evaluated the recovery of phenolic acids in plasma and urine after wheat bran consumption found only 3% of the total phenolics (soluble free, soluble conjugated, and insoluble bound) were absorbed after 24 hours. This study also concluded that the site of absorption was primarily the small intestine, with maximum absorption 1 to 3 hours after ingestion. This and other numerous studies have therefore concluded that the

low bioavailability of wheat phenolics is due to its primarily insoluble bound form in wheat bran (35, 57, 58). The results of this study showed significant conversion of insoluble bound phenolics to soluble free phenolics, therefore demonstrated potential to improve the bioaccessibility and potential bioavailability of wheat phenolic acids.

Influence of Moisture Content (MC) on Solid-State Enzymatic Reaction. Water is a required agent in hydrolysis reactions including those catalyzed by esterases and glycosidases. Amount of free water may be limited under the solid-state enzymatic reaction conditions, and thus may alter the overall effectiveness of enzyme treatments because of the reduced mobility of enzyme and reactant molecules, and the availability of water molecules as a reactant. To evaluate the potential effect MC on efficacy of enzyme treatment, Akron wheat bran was treated with Viscozyme L, a widely available and popular cell wall degrading enzyme preparation used by food manufacturers, at 30 and 43% MC levels under the solid state reaction conditions using five concentrations of enzyme. Multiple enzyme concentrations were used to obtain a general conclusion. The enzyme concentrations were from 2 to 34 units per gram dry wheat bran. The enzyme treated bran samples were extracted with ethanol and evaluated for their antioxidant properties measured as ORAC, $DPPH^{\bullet}$ scavenging capacity, and total phenolic content. MC had a significant effect on wheat bran antioxidant property changes as a result of solid-state enzyme treatments (**Figures 3.7-3.9**).

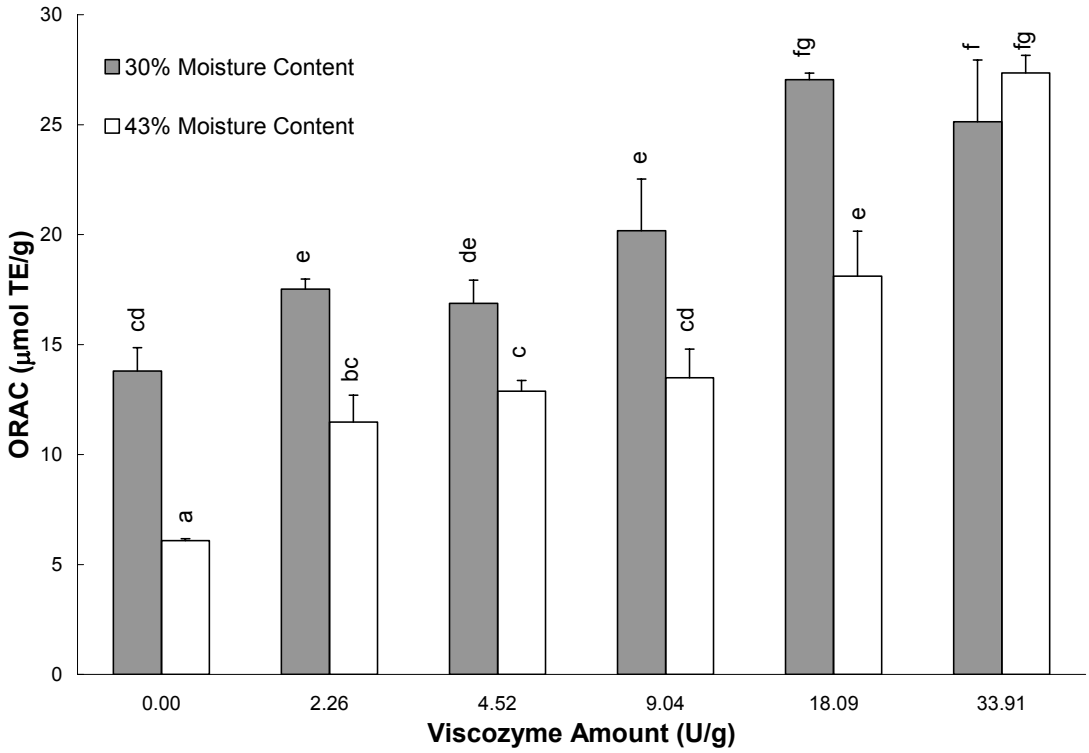

Figure 3.7. Effects of moisture content on the oxygen radical absorbing capacities (ORAC) of Akron wheat bran treated with different doses of Viscozyme L enzyme preparation. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activity for Viscozyme L. See Table 3.1 for enzyme characteristics. Results expressed as µmoles trolox equivalents per gram of wheat bran on a dry weight basis. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

Results in **Figure 3.7** indicate that Viscozyme L treatments at 30% MC resulted in significantly higher ORAC values than that at 43% MC for all tested enzyme concentrations except the highest enzyme concentration (34 U/g). The bran samples treated with 18 U/g Viscozyme L at two MCs had the largest difference in their ORAC values, which was about 1.5 fold. **Figure 3.8** indicates that at low enzyme concentration (2.0 – 4.5 U/g) bran samples treated at 30% MC had significantly higher $DPPH^{\bullet}$ scavenging capacity values than those treated with same level of enzyme at 43% MC, while MC had no significant effects when Viscozyme L

concentration was 9 and 18 U/g in the solid-reaction mixtures. At 33 U/g level, however, the bran sample treated with Viscozyme L at 43% MC had a significantly larger $DPPH^{\bullet}$ scavenging capacity (**Figure 3.8**). The TPC values of Akron bran treated with same concentrations of Viscozyme at two moisture contents were determined and compared in **Figure 3.9** on a per dry bran weight basis. Similar to that observed in **Figure 3.7** and **3.8**, the effects of moisture content on solid-state enzymatic treatments depended on the enzyme concentration in the reaction mixture. When the moisture content was 30%, bran samples treated with enzyme level of 0-4.5 U/g had significantly higher or same level of extractable TPC, whereas the bran samples treated with enzyme levels 9-34 U/g at 43% MC had higher TPC values than those reacted at 30% MC.

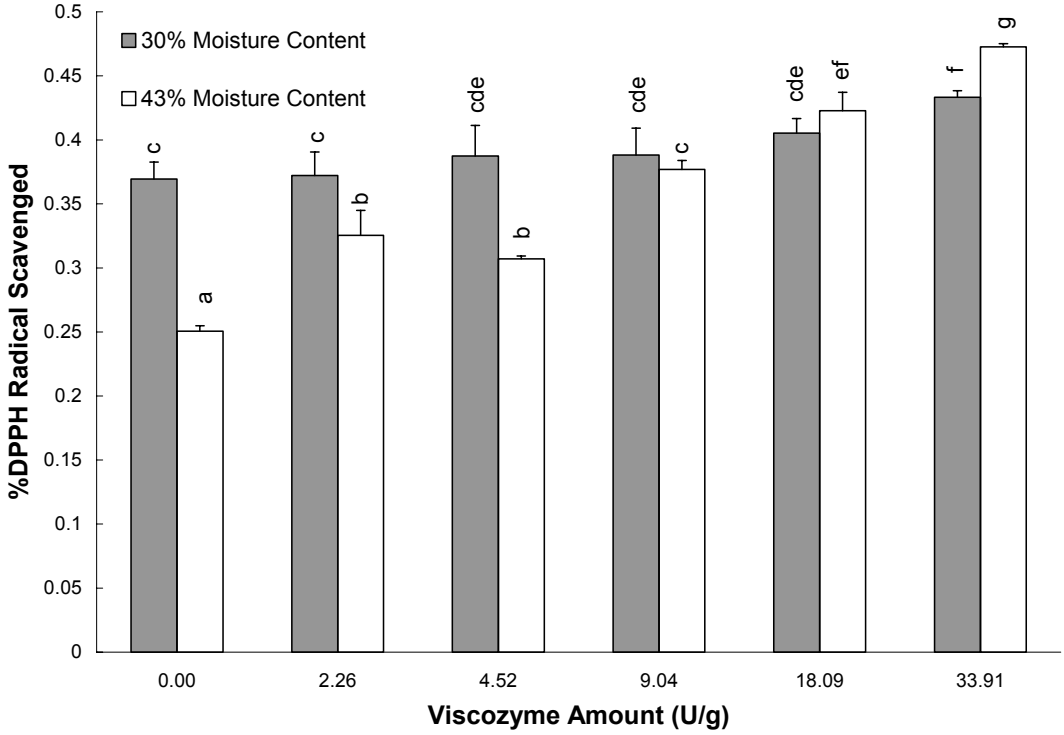

Figure 3.8. Effects of moisture content on the DPPH• scavenging capacities of Akron wheat bran treated with different doses of Viscozyme L enzyme preparation. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activity for Viscozyme L. See Table 3.1 for enzyme characteristics. Results expressed percent DPPH• scavenged after 40 minutes reaction time. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

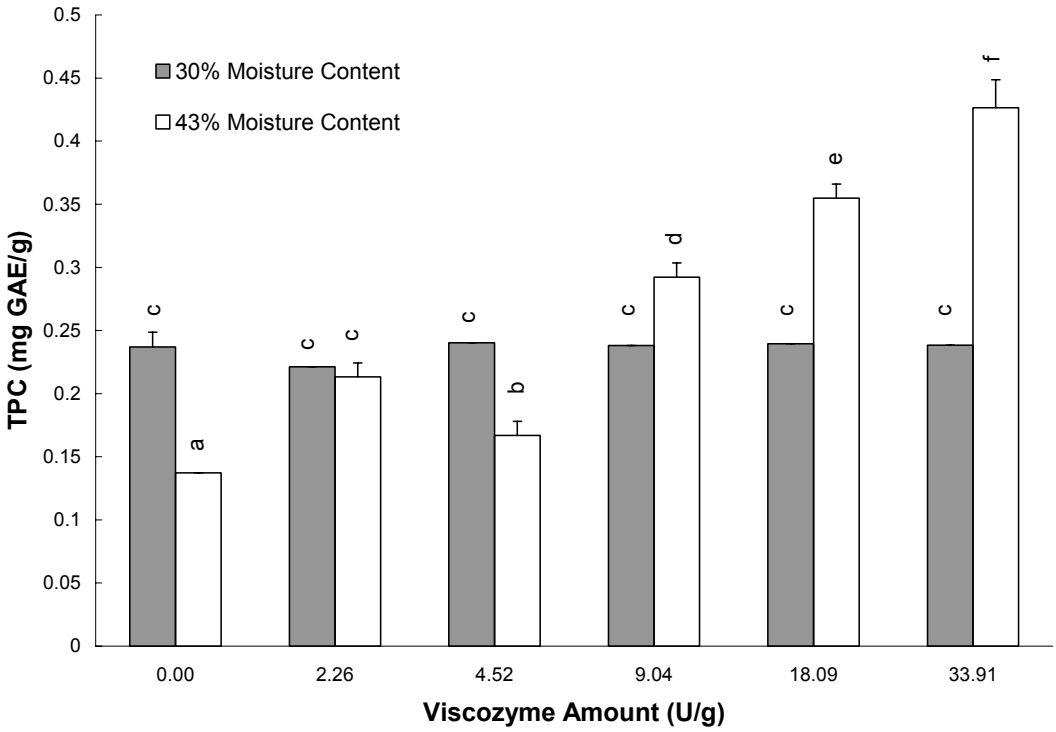

Figure 3.9. Effects of moisture content on the total phenolic contents of Akron wheat bran treated with different doses of Viscozyme L enzyme preparation. Initial reaction enzyme dosages expressed as enzyme activity units per g wheat bran on a dry weight basis, using manufacturer declared major activity for Viscozyme L. See Table 3.1 for enzyme characteristics. Results expressed mg gallic acid equivalents (GAE) per gram of wheat bran on a dry weight basis. All tests were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

Taken together the trend of these results suggest that for solid-state Viscozyme L treatments of wheat bran, the enzyme reactions are affected by moisture content in the reaction system, and the effects are dependent on enzyme concentrations. At lower enzyme levels, the solid-state enzymatic treatments at lower MC resulted in higher extractable antioxidant activities, while the opposite was true at high enzyme doses. These results may be explained on the basis that at low enzyme concentrations, less water is required as a reactant to reach the maximum velocity of the reaction and additional free water may dilute the local enzyme concentration and further reduce the maximum enzyme reaction velocity. This reduction of maximum

enzyme reaction velocity could then lead to the decrease in total extractable antioxidant activities or TPC values shown in this study when MC was increased from 30% to 43% under the solid-state enzymatic reaction conditions. On the other hand, when enzyme concentration was high in the reaction mixture, more free water was required to participate in the hydrolysis reaction. In other words, Viscozyme L was not saturated with the local free water molecules when enzyme concentration was high, and increased moisture content from 30% to 43% significantly enhanced the enzyme reaction and resulted in more extractable antioxidants. It needs to be pointed out that increasing the level of enzyme or moisture content in the solid-state enzymatic reaction system may significantly elevate the overall cost of the processing. Addition research is suggested to further understand the interaction of moisture and enzyme dose effects on improving wheat bran antioxidant properties. Further research is required to optimize the procedure before it may be utilized for commercial applications.

3.5 Conclusion

Our results showed, for the first time, that solid-state enzyme reactions can be utilized to enhance the extractable and potentially bioavailability of antioxidants in wheat bran, which may improve its health benefits as well as its commercial value and market competitiveness. The increases in antioxidant properties were shown to be dependent on both the type of enzyme preparation and its dose. This research also showed that the effects of reaction moisture content during solid-state treatments are dependent on the enzyme dosage level. The solid-state enzymatic procedure used in

this study requires no special equipments and involves no chemicals, and may have potential for commercial applications. In addition this study points to the future research opportunities in evaluating and developing more effective enzyme preparations for improving the bioaccessible antioxidants in wheat bran and other cereal grain based food and nutraceutical ingredients.

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Chapter 4: Effects of Solid-State Yeast Treatment on the Antioxidant Properties, and Protein and Fiber Compositions of Common Hard Wheat Bran

4.1 Abstract

The bran fraction of wheat grain is known to contain significant quantities of bioactive components. This study evaluated the potential of solid-state yeast fermentation to improve the health beneficial properties of wheat bran including extractable antioxidant properties, protein contents, and soluble and insoluble fiber compositions. Three commercial food grade yeast preparations were evaluated in the study along with the effects of yeast dose, treatment time, and their interaction on the beneficial components. Solid-state yeast treatments were able to significantly increase releasable antioxidant properties ranging from 28-65, 0-20, 13-19, 0-25, 50-100, 3-333% for scavenging capacities against peroxyl (ORAC), ABTS cation, DPPH and hydroxyl radicals, total phenolic contents (TPC), and phenolic acids, respectively. Yeast treatment increased protein contents 11-12%, but did not significantly alter the fiber composition of wheat bran. Effects of solid-state yeast treatment on both ORAC and TPC of wheat bran were altered by yeast dose, treatment time, and their interaction. Results suggest that solid-state yeast treatment may be a commercially viable post-harvest procedure to improve the health beneficial properties of wheat bran and other wheat-based food ingredients.

4.2 Introduction

Increasing epidemiological evidence supports the role that foods rich in antioxidants may play in preventing chronic diseases. (*1,2*). Antioxidants are thought to prevent chronic conditions by preventing damage to important biomolecules such as DNA, proteins, and lipids (*1,2*).

Wheat is an important global dietary staple with over 420 million metric tons utilized as food in 2003 (*3*). Recent studies have shown wheat to contain significant *in-vitro* antioxidant properties (*4-13*). This has included free radical scavenging activities against hydroxyl, peroxyl, superoxide anion, DPPH, and ABTS cation radicals (*4-10*). In addition, wheat has demonstrated significant antioxidative chelating activities towards reactive Fe^{2+} and Cu^{2+} metals, and the ability to prevent DNA and low-density lipoprotein oxidation (*11-13*). Phenolic acids present in wheat are thought to be a major contributor to its antioxidant properties (*14, 15*). Significant levels of phenolic acids have been detected in wheat and are thought to be mostly concentrated in the bran fraction (*8, 16, 17*). These phenolic acids might explain the health benefits of whole grain consumption found in several epidemiological studies (*14, 15*). Of recent interest, however, has been the bioavailability of wheat phenolic acids.

Phenolic acids in wheat predominately exist in an insoluble bound form, ester linked to cell wall materials in wheat bran (*5, 9, 12, 18)* Small amounts also exist in

soluble free or soluble conjugated forms (*5, 9, 12, 18)*. It is thought that absorption of wheat phenolics in the small intestine is limited to the soluble free fraction (*19*). The absorption of insoluble bound phenolic acids on the other hand, is thought to be minimal and a result of release by colonic microflora and absorption in the colon (*9, 19*). Given their predominately insoluble bound form, wheat phenolic acids are therefore thought to have low bioavailability. This has been supported by a recent *in-vivo* human study by Kern and others (*2003*). This study found that less than 3% of the ingested wheat bran phenolics (soluble bound, soluble conjugated, and insoluble bound) were absorbed over a 24 hour period in humans (*20*).

One possible strategy to improve the bioavailability of wheat bran phenolics is to release its insoluble bound phenolic acids before human consumption. This could be achieved using aqueous phase chemical, enzymatic, and/or fermentation methods. Solid-state fermentation, however, offers the advantages of being more cost and energy effective and more environmentally friendly (*21*). In addition, it requires minimal post-reaction processing to recover products (*21*). Solid-state fermentation has previously been used to release bound phenolic compounds from soybean powder, cranberry pomace, corn cobs, and pineapple waste (*22-25*). Insoluble bound phenolic acids present in wheat bran are known to be linked to arabinoxylans present in cell wall material through ester linkages (*26-27*). Previous aqueous phase studies have found cellulases, xylanases, and β-gluconases in combinations with cinnamoyl or feruloyl esterases to be effective in releasing wheat bran phenolics (*26-28*). Solid-state treatment of wheat bran with these enzymes has also been shown to be effective

in releasing wheat bran phenolics (*29*). These studies suggest that microorganism producing these enzymes under solid-state fermentation conditions could be used to enhance the release of wheat phenolics. Beyond antioxidants, the fermentation of cereals has also been shown to increase its protein contents and modify its fiber composition (*30*).

Of the reported potential microorganisms for solid-state treatments, *Saccharomyces cerevisiae* is a particularly attractive option given its GRAS (generally regarded as safe) status for food products (*31*). Strains of *S. cerevisiae* have been shown to produce enzymes including β-glucosidases, carboxylesterases, and possibly feruloyl esterases (*32-34*). In addition, *S. cerevisiae* can grow under lower water activities (A_w) than bacteria which if growing could pose a food safety issue (*21*). Together these factors may allow solid-state yeast fermentation of wheat bran to produce a product rich in natural antioxidants and dietary fiber, and enhanced protein levels. The objective of this study was therefore to test three commercially available yeast preparations for their abilities to improve the extractable antioxidant properties of wheat bran. In addition, this study evaluated these treatments for their abilities to modify the fiber and protein compositions of wheat bran. No study to date has investigated this opportunity.

4.3 Materials and Methods

Chemicals and Reagents. 2,2'-bipyridyl-2,2-diphenyl-1-picryhydrazyl radical ($DPPH^{\bullet}$), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium

salt (ABTS), fluorescein (FL), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), and the phenolic acids were purchased from Sigma-Aldrich (St. Louis, MO). 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). Three commercially available bakers yeast preparations were utilized in this study and randomly coded as Y1, Y2, and Y3. Yeast preparations included active-dry yeasts from Fleischmann (Fenton, Missouri) and Hodgson Mill (Effingham, IL), and Fleischmann's RapidRise yeast (Fenton, Missouri). All other chemicals and solvents were of the highest commercial grade and used without further purification. Ultrapure water used for all experiments was from an ELIGA Purelab Ultra Genetic polishing system with UV photo-oxidation (Lowell, MA), <5 ppb of TOC, and resistivity of 18.2 mΩ.

Common Hard Wheat Bran Samples. Bran from Lakin wheat (a hard white variety) and Akron wheat (a hard red variety) were used in this study. Both varieties were grown in Fort Collins, Colorado during the 2004 growing season under agronomic practices considered typical for wheat production in eastern Colorado. Both wheat samples were provided by Dr. Scott Haley in the Department of Soil and Crop Science, Colorado State University, Fort Collins, CO 80523. Harvested grain was cleaned using seed cleaners to remove all non-grain debris present and stored under ambient conditions. Grain samples were ground and separated into flour and bran using a Brabender Quadromat Junior experimental mill. Bran was ground to 40-mesh (420 μm) using a micro-mill manufactured by Bel Art Products (Pequannock,

NJ).

Solid-state Yeast Treatment. For all solid-state yeast treatments a known amount of yeast preparation was mixed with 4 g ground wheat bran in a sterile beaker to begin the solid-state yeast treatment. Beakers were sealed with parafilm and incubated at 32 ºC. Experiments comparing the effects of three different yeasts utilized Lakin wheat bran with a yeast preparation concentration of 0.1g per g wheat bran, and a 48 hour fermentation time. Experiments evaluating dose effects of yeast used Akron wheat bran with a 48 hour fermentation time and Y3 yeast preparation at concentrations of 0, 0.025, 0.05, 0.1, and 0.2 g per gram wheat bran. Fermentation time experiments used Akron wheat bran and Y3 yeast preparation at 0.1g per g wheat bran with fermentation times of 0, 12, 24, and 48 hours. All fermented samples were thermally inactivated using a microwave oven. Samples were then re-ground using a micro-mill to 40-mesh and stored under nitrogen at room temperature in the absence of light for further analysis. Controls were treated using the above procedures with thermally in-activated samples of the same yeast preparations used in treatments.

Sample Extraction Procedure. Treated and re-ground wheat bran samples were extracted with 100% ethanol at a ratio of 1 g material per 10 mL solvent for 18 hours under nitrogen in the dark at ambient temperature and subjected to oxygen radical absorbing capacity (ORAC), $ABTS^{\bullet+}$ scavenging capacity, and $DPPH^{\bullet}$ scavenging capacity assays. Known volumes of the 100% ethanol extracts were dried under nitrogen, and the solid residues were quantitatively re-dissolved in DMSO for

total phenolic content (TPC) assay and 100% acetone for hydroxyl radical scavenging capacity (HOSC) assay. Extractions were stored under nitrogen in the dark at ambient temperatures until further analysis.

Oxygen Radical Absorbing Capacity (ORAC) Assay. ORAC assay was conducted on the 100% ethanol extracts using fluorescein (FL) as the fluorescent probe with a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland) according to a laboratory protocol (*29*). Trolox standards were prepared in 100% ethanol while all other reagents were prepared in 75 mM sodium phosphate buffer (pH 7.4). Initial reaction mixtures contained 225 μL of 8.16×10^{-8} M FL, 25 μL of 0.36 M AAPH, and 30 μL sample, standard, or 100% ethanol for blanks. Sample and FL were mixed in 96 well plate and pre-heated for 20 minutes at 37ºC in plate reader after which the AAPH solution was added. The fluorescence of the assay mixture was recorded every minute for 80 minutes with temperature maintained at 37 ºC. Excitation and emission wavelengths were 485 nm and 535 nm respectively. Results were expressed as μmoles of trolox equivalents (TE) per g wheat bran on a dry weight basis.

Radical cation ABTS$^{\bullet+}$ scavenging capacity. The ABTS$^{\bullet+}$ scavenging capacity of the 100% ethanol extracts was evaluated according to a previously reported protocol (*7*). Working sample solutions were created by diluting 50 μL of wheat bran extracts to 500 μL with 100% ethanol. ABTS cation 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 cation radical solution with an absorbance of 0.7 at 734 nm, and 80 μL of

working sample solution or 100% ethanol for the control. The absorbance at 734 nm was measured after a reaction time of 1 minute. Trolox equivalents per gram of wheat bran on a dry weight basis were calculated using a standard curve prepared with trolox.

Radical DPPH scavenging capacity. 100% ethanol extracts were evaluated for their $DPPH^{\bullet}$ scavenging capacity determined in 96-well plates using a previously reported procedure with modifications *(9)*. Briefly, 100 μL of freshly prepared $DPPH^{\bullet}$ solution was added to 100 μL of 100% ethanol bran sample extract or 100% ethanol for blank and absorbance was measured at 515 nm every minute for 1 hour using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). The initial concentration of $DPPH^{\bullet}$ was 100 μM for all reaction mixtures. $DPPH^{\bullet}$ radical scavenging capacity was expressed as the percent of $DPPH^{\bullet}$ scavenged at 40 minutes of reaction.

Hydroxyl Radical Scavenging Capacity (HOSC) Assay. 100% acetone extracts were evaluated for their hydroxyl radical scavenging capacity according to a previously published protocol (*10*) using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). Reaction mixtures consisted of 170 μL of 9.28×10^{-8} M FL prepared in 75 mM sodium phosphate buffer, 30 μL of standard or sample or solvent blank, 40 μL of 0.1990 M H_2O_2, and 60 μL of 3.43 mM $FeCl_3$. Fluorescence was measured every minute for 3 hours with an excitation wavelength of 485 nm and emission wavelength of 535 nm. Trolox standards were prepared in 100% acetone

and a standard curve was used for HOSC quantification. HOSC values were expressed as micromoles of trolox equivalents (TE) per gram of wheat bran on a dry weight basis.

Total Phenolic Contents. The total phenolic contents of 100% DMSO extracts were determined using a previously reported procedure with the Folin-Ciocalteu reagent (*9*). Folin-Ciocalteu reagent was prepared by refluxing sodium molybdate, sodium tungstate, 85% phosphoric acid, and concentrated hydrochloric acid for 10 h, reacting with lithium sulfate, then oxidizing with bromine followed by filtering. The final reaction mixture contained 250 µL freshly prepared Folin-Ciocalteu reagent, 750 µL 20% sodium carbonate, 3 mL of ultra-pure water, and 50 µL of 100% DMSO extracts. Absorbance at 765 nm was read after a reaction time of 2 h at ambient temperature. Total phenolic contents were calculated using gallic acid as a standard and expressed as mg gallic acid equivalents (GAE) per g wheat bran on a dry weight basis.

Phenolic Acid Composition. Samples were analyzed for their soluble free, soluble conjugated, insoluble bound, and total phenolic acid compositions using a laboratory procedure previously reported (*9*). Briefly, acetone/methanol/water (7/7/6, v/v/v) was used to extract the soluble free and the soluble conjugated phenolic acids, while the insoluble phenolic acids in the residue were released by NaOH hydrolysis followed by neutralization and solvent extraction. The free and conjugated phenolic acids in the acetone/methanol/water solution were separated based on their solubility under acidic condition (pH 2) by extracting soluble free phenolic acids into ethyl

acetate and ethyl ether (1:1, v/v). Soluble conjugated phenolic acids were also hydrolyzed in the presence of NaOH. 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 and analyzed by HPLC using a C18 Phenomenex column (250 mm × 4.6 mm) according to an established protocol (*26*). Phenolic acids were separated with 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). Solvent gradient was programmed from 10 to 100% B in 42 min with a flow rate of 1.0 mL/min (*9*). Identification of phenolic acids was accomplished by comparing standards to the retention time of peaks in the MeOH solutions. Quantification of each phenolic acid was performed using external standards and total area under each peak.

Protein Analysis. Protein content of samples was determined using the crude protein combustion AACC Method 46-30 (*35*). About 150 mg of each sample was used for analysis using LECO FP-528 protein determinator (LECO Corporation, St. Joseph, MI). Percent protein values were calculated on a dry weight basis using a protein factor of 6.25.

Fiber Analysis. Insoluble and soluble fiber contents in the fermented wheat bran samples were determined and compared to that of the control bran samples which went through the fermentation process without active yeast using a commercial total dietary fibre assay kit from Megazyme International Ireland Ltd (Wicklow, Ireland). The enzymatic assay was conducted according to a laboratory protocol (*36*)

based on the AACC Method 32-07 (*35*).

Moisture Content. The moisture content of bran samples was determined using the air-oven aluminum plate method AACC 44-16 (*35*).

Statistical Analysis. Treatment and measurement replication indicated in figure captions. ANOVA and Tukey's tests performed (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL) to identify differences among means, with statistical significance was declared at $P \leq 0.05$.

4.4 Results and Discussion

Effects of Three Commercial Food Grade Yeasts on the Antioxidant Properties of Lakin Wheat Bran Under Solid-state Fermentation Conditions. Solid-state yeast treatments for wheat bran were carried out using three commercially available bakers yeast preparations. Controls used thermally inactivated samples of the same yeasts used in treatments. The three yeasts used in this study are represented as Y1, Y2 and Y3. Solid-state treatment conditions including temperature, moisture content, and yeast to bran ratio were selected according to the results of preliminary experiments using Lakin wheat bran. Individual yeast-treatments were found to differ in their abilities to improve the evaluated antioxidant properties of wheat bran.

Oxygen Radical Absorbing Capacities (ORAC) – ORAC measures peroxyl radical scavenging capacity. Results for ORAC were expressed as μmoles of trolox equivalents per gram wheat bran on a dry weight basis. All yeasts tested were able to

significantly increase ORAC values compared to the controls (**Figure 4.1)**. Y2 treatment demonstrated the greatest increase in ORAC versus the control sample showing a 65% increase in ORAC value. Treatments for Y3 and Y1 showed ORAC increases of 28 and 36%, respectively, under the experimental conditions. These data suggest the possible application of solid-state yeast treatment for improving the releasability and potential bioavailability of wheat bran antioxidants. These data also indicate that bran-based food ingredients may be produced with optimized yeast preparations and fermentation conditions, which warrants further research activities. It needs to be pointed out that the ORAC values for all three controls were also significantly different (**Figure 4.1**). This may be explained by the fact that the yeast preparations might contain different levels of peroxyl radial scavenging agents, and also suggests the importance of yeast preparation in the quality and value of the fermented wheat bran products.

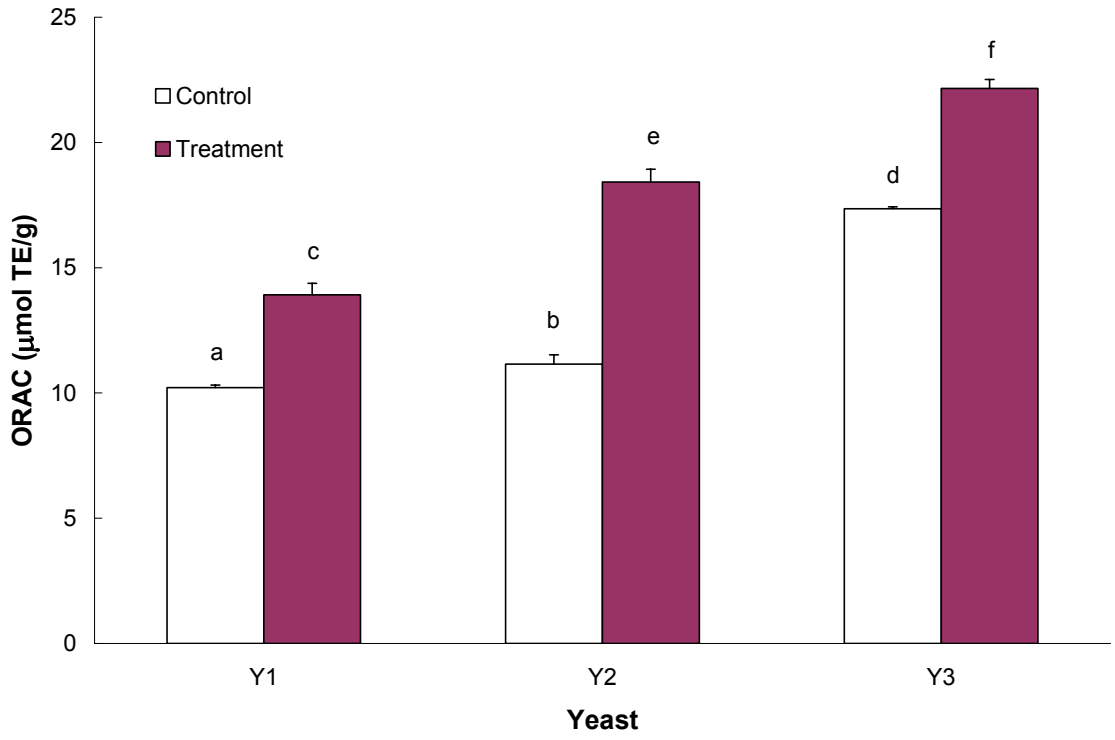

Figure 4.1. Effect of solid-state fermentation with different yeasts on the oxygen radical absorbing capacities of Lakin wheat bran. Solid-state fermentation conditions included yeast concentration of 0.1g yeast per g bran, fermentation time of 48 hours, and temperature of 32ºC. Results expressed as µmoles trolox equivalents per gram of wheat bran on a dry weight basis. All measurements were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

ABTS$^{\bullet+}$ Scavenging Capacities — The ABTS$^{\bullet+}$ scavenging capacities of the fermented bran samples were analyzed and compared to that of the controls (**Figure 4.2**). In contrast to ORAC results, Y1 and Y2 treatments showed no significant effects compared to corresponding controls for ABTS$^{\bullet+}$ scavenging capacities. Treatment with Y3 did, however, show a 20% increase for ABTS$^{\bullet+}$ scavenging capacity. Controls for all three yeasts did not show significant differences indicating

that the yeasts had similar $ABTS^{\bullet+}$ scavenging components or they did not contain significant level of such chemicals. Results from both ORAC and $ABTS^{\bullet+}$ scavenging capacity data indicate that estimations for the effects solid-state yeast fermentation on wheat bran antioxidant properties are dependent the antioxidant activity assay utilized. The use of a single antioxidant capacity assay is therefore not adequate for developing these procedures to improve the releasability of wheat antioxidants.

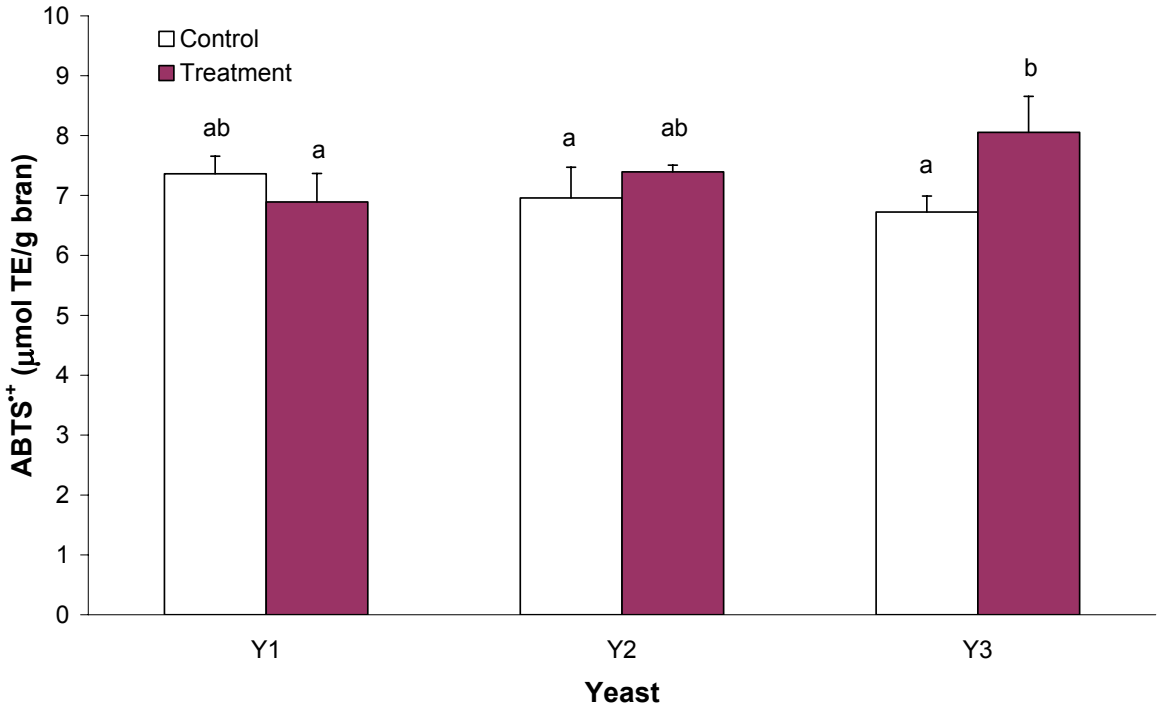

Figure 4.2. Effect of solid-state fermentation with different yeasts on the $ABTS^{\bullet+}$ scavenging capacities of Lakin wheat bran. Solid-state fermentation conditions included yeast concentration of 0.1g yeast per g bran, fermentation time of 48 hours, and temperature of 32ºC. Results expressed as µmoles trolox equivalents per gram of wheat bran on a dry weight basis. All measurements were conducted in triplicate, and mean values are reported The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

DPPH$^{\bullet}$ Scavenging Capacities – **Figure 4.3** compares the % DPPH radicals scavenged by each sample evaluated on a same per bran dry weight basis. Results indicated a significant decrease of 17% in DPPH radical scavenging capacity versus control for Y1-fermented bran sample. Treatments with Y2 and Y3, however, showed increases in DPPH radical scavenging activity versus the control of 13 and 19% respectively. Similar to ORAC results, the controls were significantly different, indicating that the yeasts themselves might have different DPPH$^{\bullet}$ scavenging capacities.

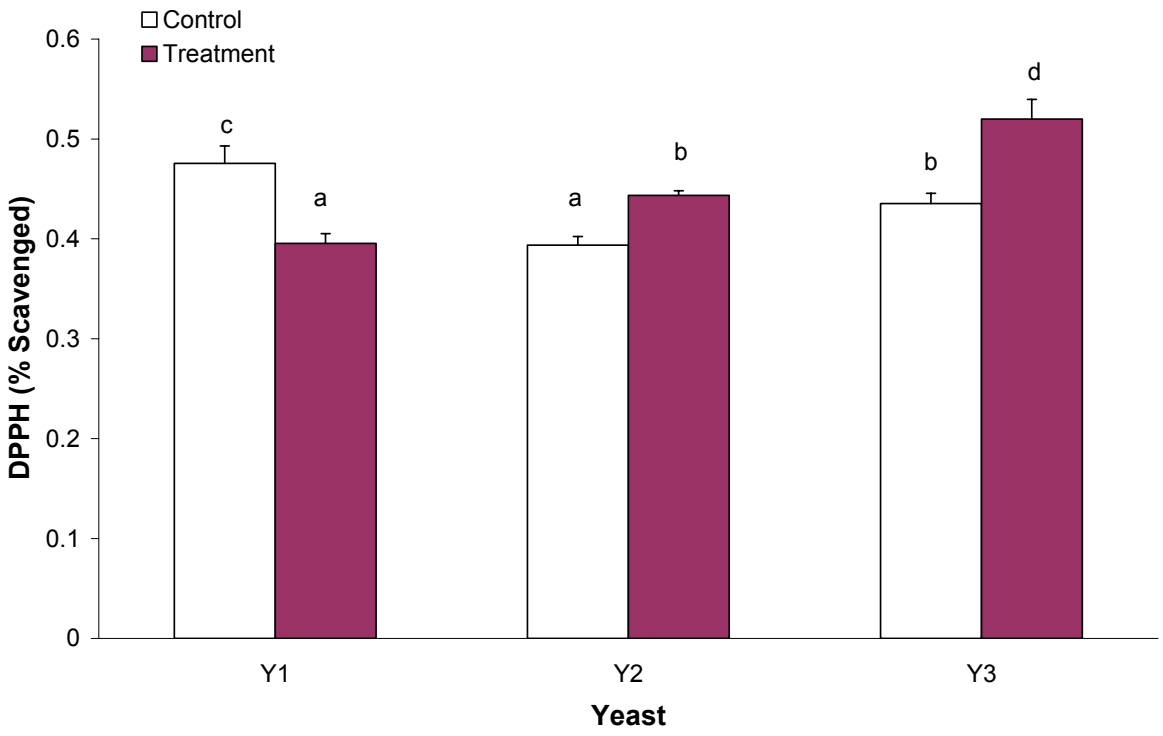

Figure 4.3. Effect of solid-state fermentation with different yeasts on the DPPH radical scavenging capacities of Lakin wheat bran. Solid-state fermentation conditions included yeast concentration of 0.1g yeast per g bran, fermentation time of 48 hours, and temperature of 32ºC. Results expressed percent DPPH$^{\bullet}$ scavenged after 40 minutes reaction time. All measurements were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

Hydroxyl Radical Scavenging Capacities (HOSC) – **Figure 4.4** shows the hydroxyl radical scavenging capacity treatment and control samples using a Fe^{3+}/H_2O_2 hydroxyl radical generating system. Similar to the $ABTS^{\bullet+}$ scavenging capacity results, Y3 treatment resulted in a significant 25% increase in hydroxyl radical scavenging activity versus the control. Treatments with Y1 and Y2 treatments showed no significant increases in activity versus the corresponding controls.

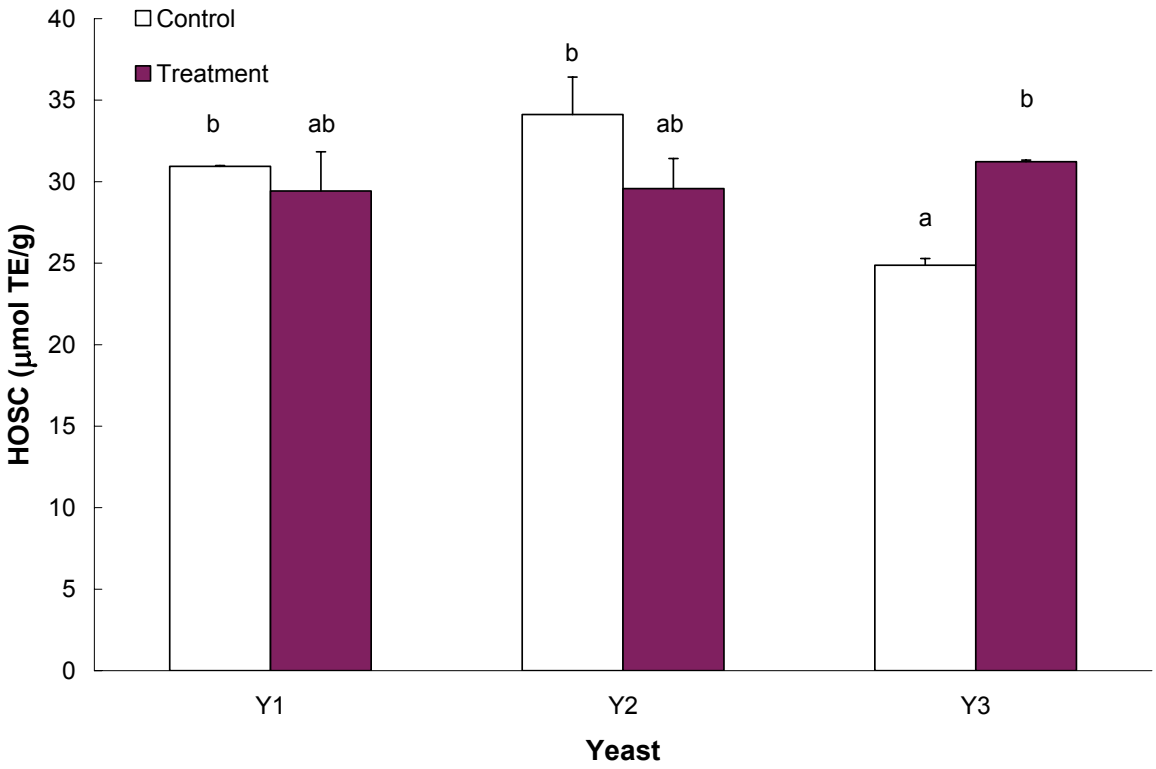

Figure 4.4. Effect of solid-state fermentation with different yeasts on the hydroxyl radical scavenging capacities of Lakin wheat bran. Solid-state fermentation conditions included yeast concentration of 0.1g yeast per g bran, fermentation time of 48 hours, and temperature of 32ºC. Results expressed as µmoles trolox equivalents per gram of wheat bran on a dry weight basis. All measurements were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

Total Phenolic Contents (TPC) – **Figure 4.5** shows the total phenolic contents of the control and the yeast treated bran samples determined using the Folin-Ciocalteu reagent. All three yeast treatments increased the extractable phenolic contents of Lakin wheat bran. Increases in TPC of 50, 100, and 69% versus controls were observed for Y1, Y2, and Y3 respectively. It was interesting that the yeasts were most effective at increasing extractable TPC compared to other extractable

antioxidant properties examined. The controls also differed in their TPC values indicating that the three tested yeasts may have different level of phenolics.

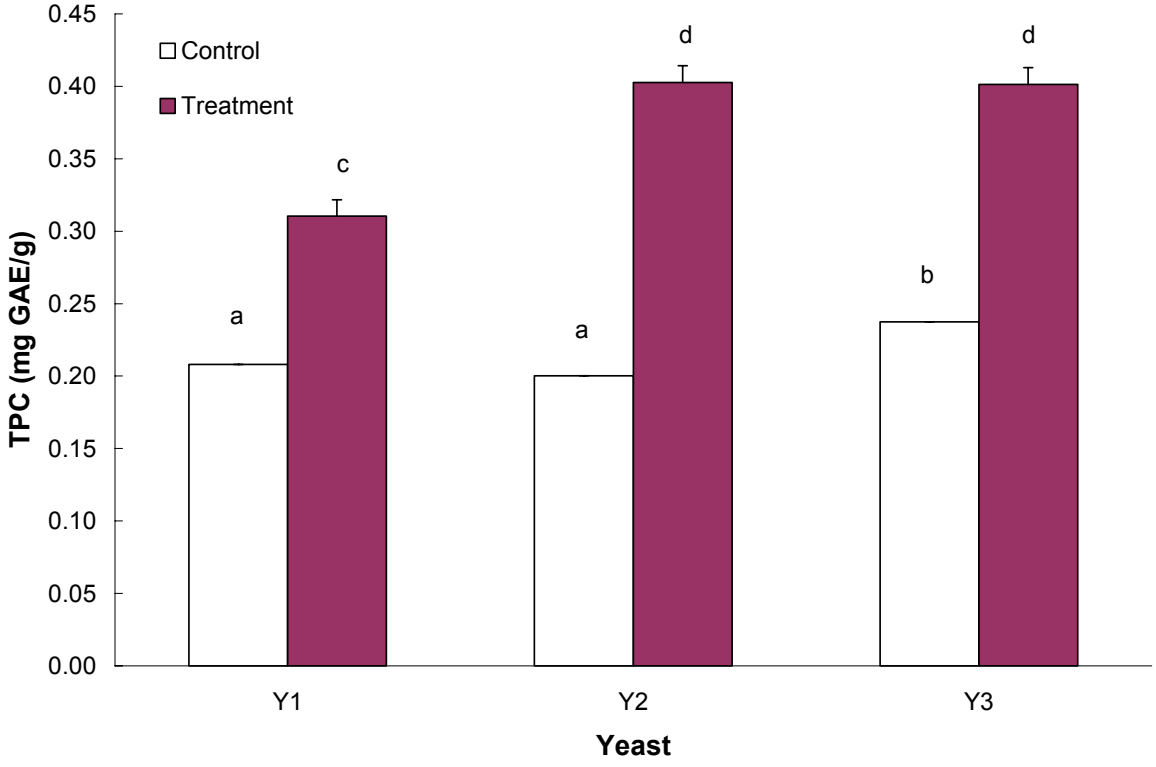

Figure 4.5. Effect of solid-state fermentation with different yeasts on the total phenolic content of Lakin wheat bran. Solid-state fermentation conditions included yeast concentration of 0.1 g yeast per g bran, fermentation time of 48 hours, and temperature of 32 ºC. Results expressed mg gallic acid equivalents (GAE) per gram of wheat bran on a dry weight basis. All measurements were conducted in duplicated, and mean values are reported. 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 Compositions – The soluble free phenolic acid compositions of all three yeast treated bran samples and controls were evaluated using HPLC. These results were expressed in μg phenolic acid per g wheat bran on a dry weight basis. Results in **Figure 4.6** show that four soluble free phenolic acids were detected in all samples evaluated, including vanillic, syringic, *p*-coumaric, and ferulic acids. The

concentrations of soluble free syringic, *p*-coumaric, and ferulic acids showed significant increases versus the controls for all three yeasts with increases ranging from 183-333%, 30-48%, and 3-51% respectively for these three phenolic acids. This indicates that all three yeasts may produce hydrolytic enzymes capable of releasing soluble conjugated or insoluble bound phenolic acids from wheat bran. In contrast, soluble free vanillic acid concentrations showed significant decreases versus controls of 75, 76, and 43% for Y1, Y2, and Y3 treated bran samples, respectively. This decrease indicates that these yeasts may be able to convert vanillic acid to other compounds through enzymatic reactions. In addition, the vanillic acid concentrations in controls significantly differed ranging from 35 to 82 μg/g bran, indicating that some of the evaluated yeasts contained this phenolic acid in the soluble free form. Interestingly, strains of *S. cerevisiae* has been reported to have a variety of phenolic acid biotransformation activities involving ferulic and vanillic acid derivatives (*37)*. This may partially explain the changes in soluble free phenolics observed in this study.

Solid-state treatment with Y3 was further analyzed for its capacity to alter wheat bran soluble conjugated, and insoluble bound phenolics. Y3 was chosen for further analysis as it showed significant changes in soluble free concentrations of all phenolic acids detected. Results in **Figure 4.7**, show that treatment of wheat bran with Y3 significantly altered soluble conjugated and insoluble bound concentrations for most of the four phenolic acids detected (vanillic, syringic, *p*-coumaric, and ferulic acids). Y3 treatment significantly decreased insoluble bound concentrations

for all four phenolic acids detected versus controls. Soluble conjugated phenolic acid concentrations were significantly increased as result of Y3 treatment, except for *p*-coumaric acid where no change was observed. These results suggest that Y3 may have produced enzymes capable of releasing all four detected insoluble bound phenolic acids in wheat bran thereby increasing its soluble free and or soluble conjugated phenolic acid contents.

Together, these data indicate that solid-state fermentation of wheat bran with yeast has the potential to increase its extractable and potentially bioavailable antioxidant properties. Changes in these properties due to treatments differed among the three yeast preparations tested. Treatment with Y3 showed the most potential, significantly increasing most extractable antioxidant properties and soluble free concentration of phenolic acids. Y2 showed some significant increases in some antioxidant properties and no change from control for other properties. Y1 treatments showed increases and decreases depending on antioxidant property examined. These results indicate that yeast preparation type plays an important role in the effectiveness of these solid-state yeast treatments for improving wheat bran antioxidant properties. It should be noted that beyond the strains of yeast used in different yeast preparations, other functional ingredients added to these preparations may alter antioxidant properties.

Results from this study also found that some insoluble bound and soluble conjugated phenolic acids remained after the tested treatments. This indicates that potential exists to further improve the hydrolytic efficiency of these solid-state

treatments and thereby further improve antioxidant properties of wheat bran. This could involve identifying other safe microorganisms (including recombinant yeasts) capable of enzymatically hydrolyzing the arabinoxylan wheat bran matrix to which phenolic acids are bound. Xylanases in particular, are a type of enzyme missing from *S. cerevisiae* which are important for phenolic acid release from this matrix (*26-28*). Many studies have reported insertion of genes encoding these enzymes from other microorganisms into *S. cerevisiae*, which may serve as one of the possible future approaches (*31, 38*). Also noted is that food-grade xylanase preparations are commercially available. Used in combined solid-state enzymatic and yeast treatments these xylanases may another potential approach to further enhance the releasability of wheat bran antioxidants.

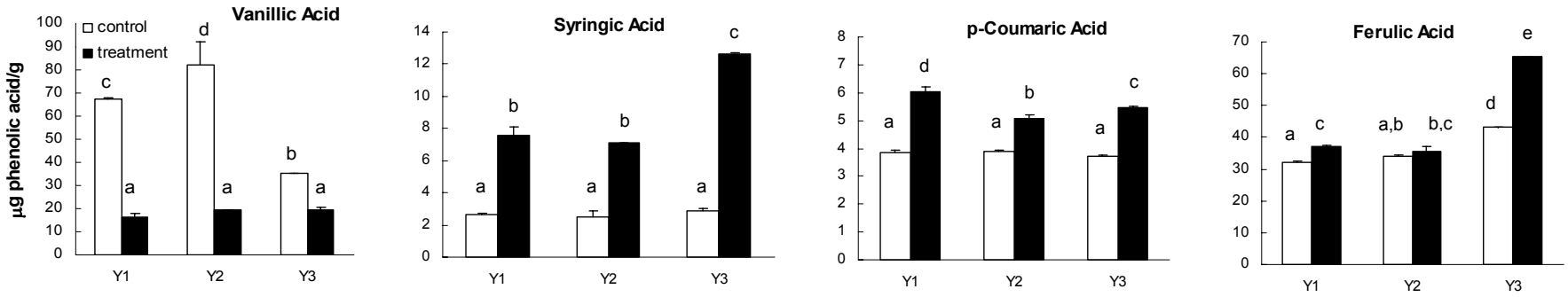

Figure 4.6. Effect of solid-state fermentation with different yeasts on the soluble free phenolic acid contents of Lakin wheat bran. Solid-state fermentation conditions included yeast concentration of 0.1g yeast per g bran, fermentation time of 48 hours, and temperature of 32ºC. Results expressed µg individual phenolic acids per gram of wheat bran on a dry weight basis. All tests were conducted in duplicated, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

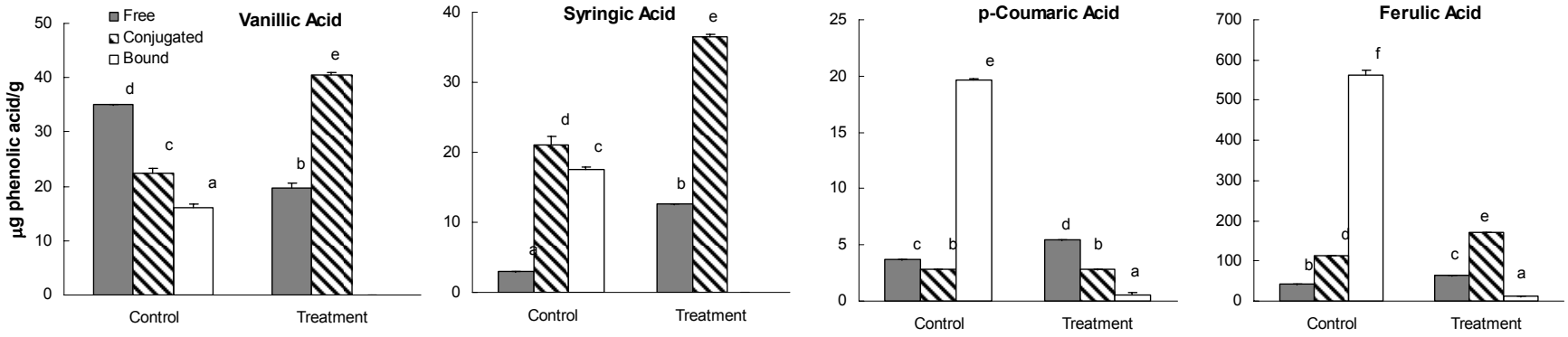

Figure 4.7. Effect of solid-state fermentation with Y3 yeast on the phenolic acid composition of Lakin wheat bran. Solid-state fermentation conditions included yeast concentration of 0.1 g yeast per g bran, fermentation time of 48 hours, and temperature of 32 ºC. Free stands for soluble free; conjugated stands for soluble conjugated; bound stands for insoluble bound. Results expressed µg individual phenolic acids per gram of wheat bran on a dry weight basis. All measurements were conducted in duplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

Effects of Solid-state Fermentation on the Protein and Fiber Composition of Lakin Wheat Bran using Three Yeasts. To evaluate the effects of the solid-state yeast fermentations on the fiber composition of wheat bran, the soluble and insoluble fiber contents of treated and control bran samples were determined. Results in **Table 4.1** indicate that Y3 significantly increased the insoluble fiber contents (10%) but had little effect on the soluble fiber content. Y1 and Y2 treatments had no significant effects for either fiber fraction. The effect on total dietary fiber shown in **Table 4.1** indicates that Y3 and Y2 slightly increased total fiber (5%) while Y1 had no significant effect. These data suggest that individual yeasts may differ in their ability to modify soluble and insoluble fibers in the tested solid-state reaction systems. This may be explained by the fact that individual yeasts may produce different enzymes. These enzymes may interact differently with soluble and insoluble fiber components, although the exact mechanism was not clear.

Table 4.1. Effect of solid-state fermentation with different yeasts on the fiber contents of Lakin wheat bran*

Yeast		IDF (g/100g)	SDF (g/100g)	TDF (g/100g)
Y1	Control	29.35 ± 0.08a	3.68 ± 0.34a	33.04 ± 0.26a
	Treatment	29.26 ± 0.86a	4.09 ± 0.73a	33.35 ± 0.12ab
Y2	Control	28.33 ± 0.48a	4.55 ± 0.50ab	32.89 ± 0.98a
	Treatment	29.54 ± 0.10a	5.31 ± 0.10ab	34.85 ± 0.20bc
Y3	Control	28.21 ± 0.61a	5.95 ± 0.32b	34.16 ± 0.29ab
	Treatment	31.25 ± 0.17b	4.93 ± 0.36ab	36.18 ± 0.18c

IDF stands for insoluble dietary fiber contents; SDF stands for soluble dietary fiber content; TDF stands for total dietary fiber content.

*Solid-state fermentation conditions included yeast concentration of 0.1g yeast per g bran, fermentation time of 48 hours, and temperature of 32ºC. Results expressed as percent fiber (g fiber per g wheat bran) on a dry weight basis. All tests were conducted in duplicate, and mean values are reported. Values marked by the same letter are not significantly different ($P < 0.05$).

The total protein contents of treatment and control samples were determined and shown in **Figure 4.8**. Results indicated that all three tested solid-state yeast treatments of wheat bran significantly increased protein contents versus controls. Increases between 11 and 12% versus the controls were observed. No significant difference was found between protein levels among bran samples treated with the

three different yeasts, or between controls. This indicates that all the three yeasts behaved similarly in increasing protein contents. The observed increases were likely the result of yeast proliferation since the solid-state treatments were not carried out under strictly anaerobic conditions. This might allow increases in total cell number and therefore protein concentration. Wheat bran is known to have moderate protein digestibility, and in terms of amino acid composition is limiting with respect to lysine (*30*). Previous studies have shown that fermentation was able to significantly improve both the lysine content and protein digestibility of cereals including wheat (*30*). In addition, these previous studies have also shown that fermentation time and temperature significantly alter these changes (*30*). It would therefore be interesting for future studies to evaluate the effects of solid-state yeast fermentations on the protein quality of wheat bran. In addition, optimal fermentation parameters such as aeration and oxygen control should be investigated for their effects on protein changes.

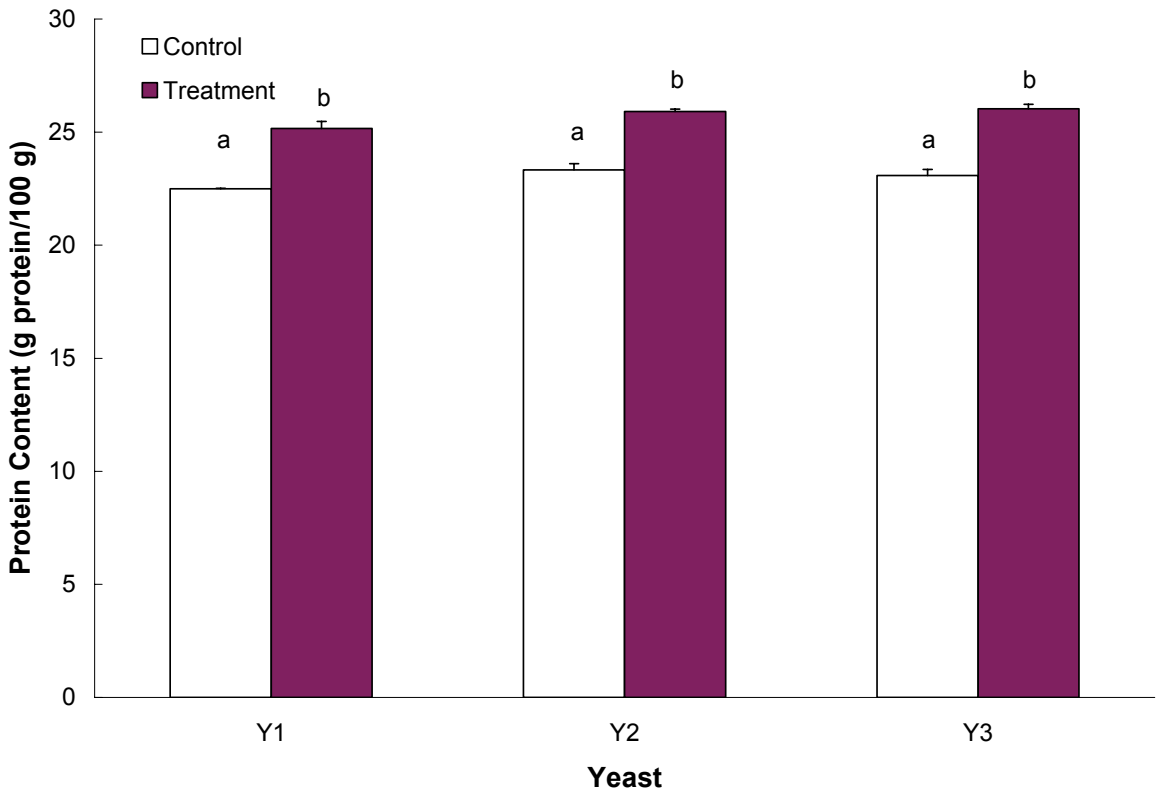

Figure 4.8. Effect of solid-state fermentation with different yeasts on the protein contents of Lakin wheat bran. Solid-state fermentation conditions included yeast concentration of 0.1 g yeast per g bran, fermentation time of 48 hours, and temperature of 32 ºC. Results expressed as percent protein (g protein per g wheat bran) on a dry weight basis. All measurements were conducted in duplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

Effects of Yeast Dose and Fermentation Time on the Antioxidant Properties of Akron Wheat Bran.

Two experiments were carried out to determine the effects of initial yeast preparation dose and fermentation time in improving releasable wheat bran antioxidant properties. The most promising one of the three yeasts evaluated, Y3, was utilized in these experiments. Antioxidant properties evaluated in these experiments included ORAC and TPC. Bran of a different wheat variety, Akron, was used in these experiments so that the conclusion from this

research on solid-state yeast treatment of wheat bran could be applied to wheat in general.

The effect of fermentation time was evaluated at times of 0, 12, 24, and 48 hours using a yeast preparation concentration of 0.1 g yeast per gram bran. Controls were run under same conditions using thermally inactivated yeast. Results for both ORAC and TPC (**Figures 4.9 and 4.10**) show a fermentation time dependent effect on antioxidant properties of solid-state yeast treated wheat bran. Results for ORAC (**Figure 4.9**) show a significant increase (24%) in ORAC from time 0 to 12 hours. A decrease in ORAC was observed from 12 to 24 hours followed by an increase from 24 to 48 hours (**Figure 4.9**). ORAC results for controls indicate a decrease in ORAC from time 0 to 12 hours followed by an increase from 12 to 48 hours. Results for TPC (**Figure 4.10**) show a similar trend with the greatest percent increase versus a control occurring after 12 hours of fermentation. The overall trend of these ORAC and TPC results show an increase in extractable antioxidant properties for solid-state fermented wheat bran over time. The most rapid changes occurred during the first twelve hours with a possible drop between 12 and 24 hours. These results can be explained in that during the entire time course, yeast cells could be producing enzymes capable of hydrolyzing bound antioxidative compounds in wheat bran. This could result in increased extractable antioxidant properties. In addition, the most rapid increases observed, occurring from 0 to 12 hours, are possibly when yeast cells are possibly proliferating at the greatest rate due to nutrient availability.

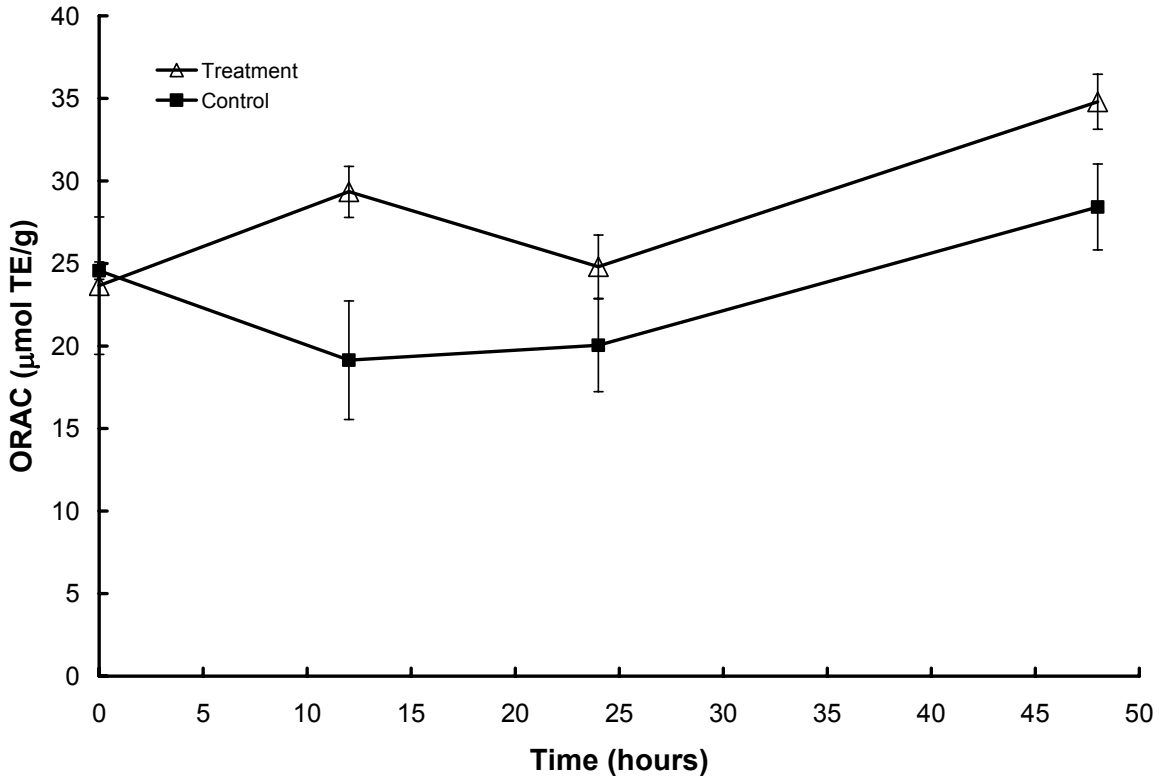

Figure 4.9. Effect of fermentation time on the oxygen radical absorbing capacities of Akron wheat bran using yeast Y3. Solid-state fermentation conditions included yeast concentration of 0.1 g yeast per g bran, fermentation times of 0, 12, 24, and 48 hours, and temperature of 32 ºC. Results were expressed as µmoles trolox equivalents per gram of wheat bran on a dry weight basis. Treatments were conducted in triplicate, controls were conducted in duplicate, and measurements for both were made in duplicate. Mean values are reported. The vertical bars represent the standard deviation of each data point.

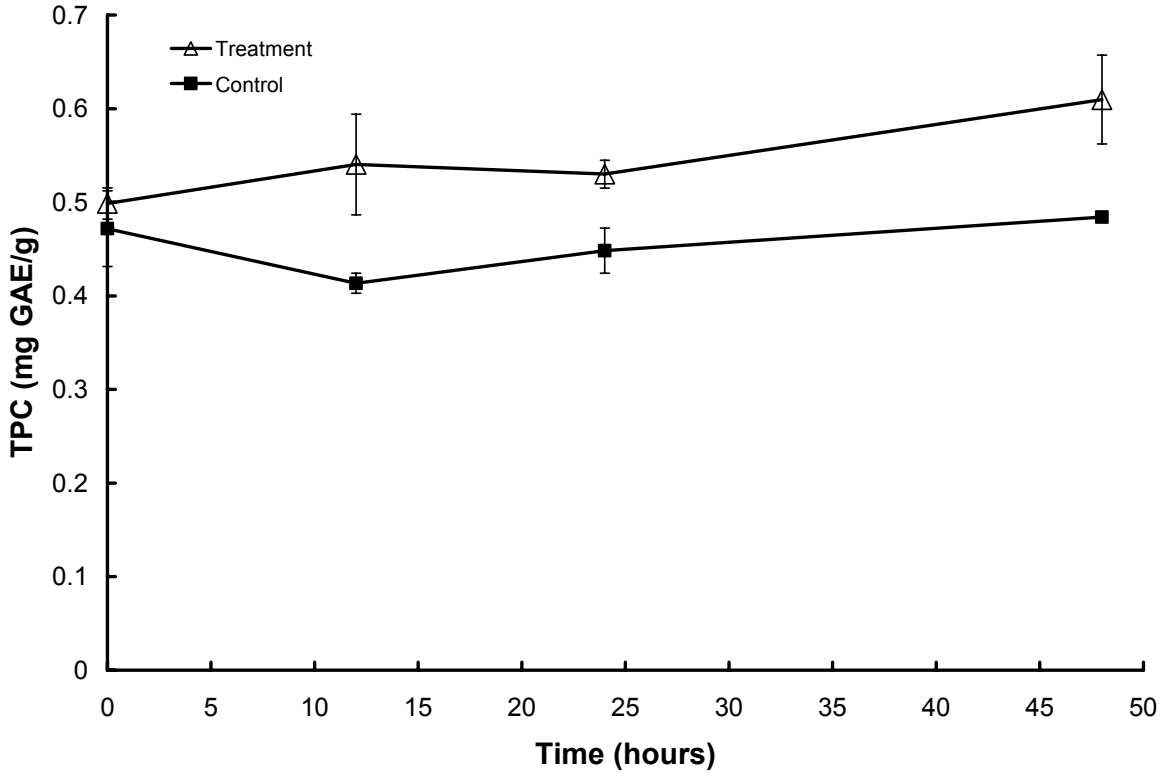

Figure 4.10. Effect of fermentation time on the total phenolic contents of Akron wheat bran using yeast Y3. Solid-state fermentation conditions included yeast concentration of 0.1g yeast per g bran, fermentation times of 0, 12, 24, and 48 hours, and temperature of 32 ºC. Results expressed mg gallic acid equivalents (GAE) per gram of wheat bran on a dry weight basis. Treatments were conducted in triplicate, controls were conducted in duplicate, and measurements for both were made in duplicate. Mean values are reported. The vertical bars represent the standard deviation of each data point.

The effect of yeast dose was evaluated using initial fermentation Y3 preparation concentrations of 0, 0.025, 0.05, 0.1, and 0.2 g per g of wheat bran. These fermentations were carried out with a fermentation time of 48 hours. Results shown in **Figures 4.11 and 12** for ORAC and TPC suggest that increases in wheat bran antioxidant activities as a result of yeast fermentation are dose dependent. **Figure 4.11** shows a dose dependent increase in antioxidant capacity for concentrations up to 0.05 g yeast per g bran. This included a 52% increase at 0.05 g

yeast per g bran compared to the control without active yeast (**Figure 4.12**). All control samples had no significant difference in their ORAC values indicating that the thermally inactivated yeast preparation itself (Y3) did not contribute significant peroxyl radical scavenging properties (**Figure 4.11**). Y3 treatments dose-dependently enhanced the availability of TPC results (**Figure 4.12**) with an 83% increase in TPC value at a dose of 0.2 g yeast per g bran compared to the corresponding control containing no active yeast. Also similar to ORAC results, controls for TPC did not show any dose dependent changes, indicating that thermally inactivated Y3 did not contribute significant phenolic contents. Together these results demonstrate that changes in wheat bran antioxidant properties as a result of solid-state yeast treatment are both time and dose dependent. Further research should be undertaken to better understand the time-course of these fermentations using more time intervals. In addition, research evaluating the effects of moisture content, particle size, aeration, and atmospheric conditions should be performed to optimize conditions for these solid-state yeast fermentations.

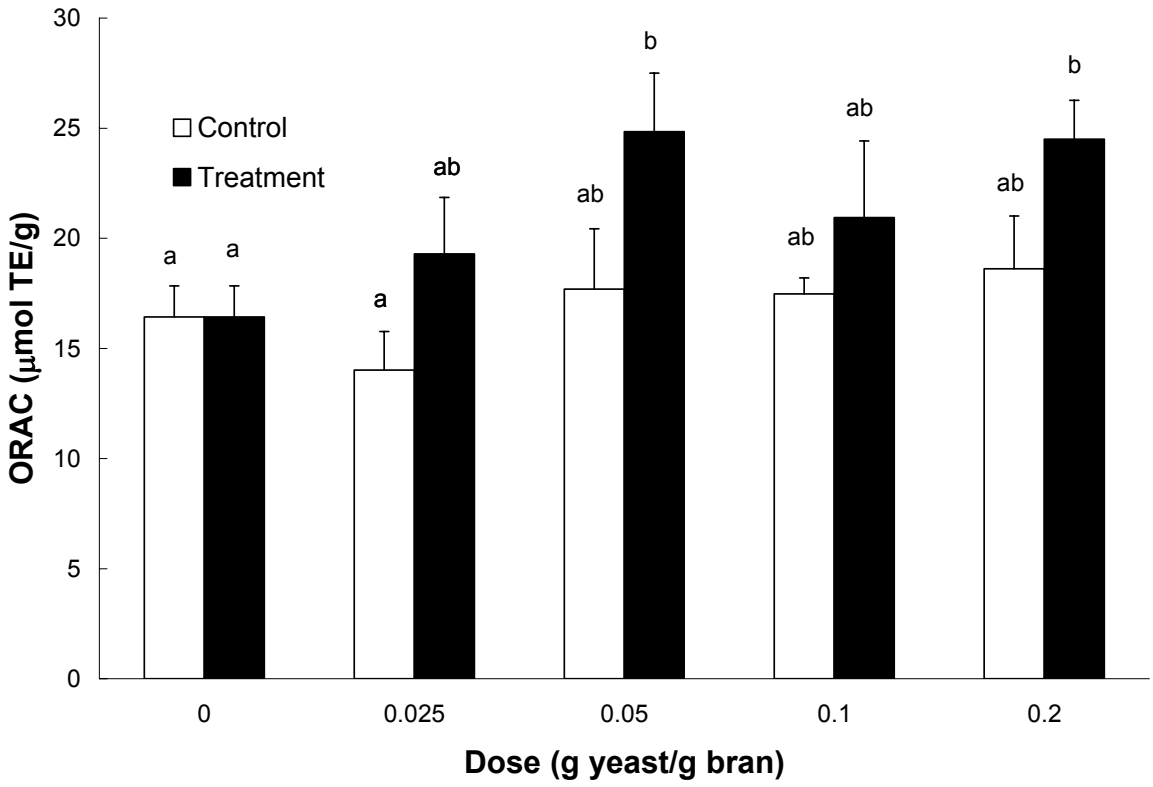

Figure 4.11. Effect of yeast dose on the oxygen radical absorbing capacities of Akron wheat bran using yeast Y3. Solid-state fermentation conditions included yeast concentration of 0, 0.025, 0.05, 0.1, and 0.2 g yeast per g bran, fermentation times of 48 hours, and temperature of 32 ºC. Results expressed as μmoles trolox equivalents per gram of wheat bran on a dry weight basis. Treatments were conducted in triplicate, controls were conducted in duplicate, and measurements for both were made in duplicate. Mean values are reported. The vertical bars represent the standard deviation of each data point.

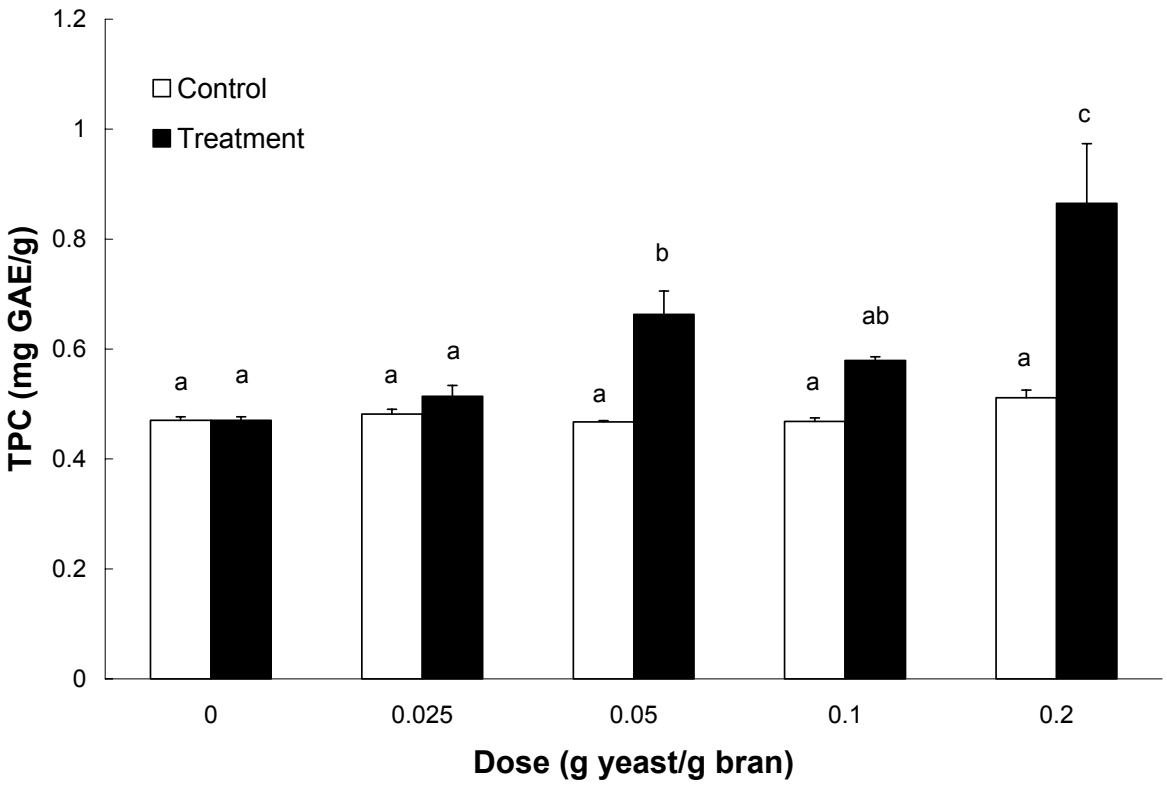

Figure 4.12. Effect of yeast dose on the total phenolic contents of Akron wheat bran using yeast Y3. Solid-state fermentation conditions included yeast concentration of 0, 0.025, 0.05, 0.1, and 0.2 g yeast per g bran, fermentation times of 48 hours, and temperature of 32 ºC. Results expressed mg gallic acid equivalents (GAE) per gram of wheat bran on a dry weight basis. Treatments were conducted in triplicate, controls were conducted in duplicate, and measurements for both were made in duplicate. Mean values are reported. The vertical bars represent the standard deviation of each data point.

4.5 Conclusion

In summary, the present study demonstrates for the first time the potential of solid-state yeast fermentation for improving extractable and potentially bioavailable wheat bran antioxidant properties, and increasing its protein and fiber contents. This study suggests that solid-state yeast treatment conditions influence the changes in these properties for wheat bran samples. Beyond wheat bran, the yeast solid-state yeast treatment procedures developed in this study may have potential application to

improve the bioavailable nutraceutical and nutritional properties of other cereal and nutraceutical ingredients.

4.6 Literature Cited

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Chapter 5: Effects of Baking Conditions, Dough Fermentation, and Bran Particle Size on the Antioxidant Properties of Whole-Wheat Pizza Crust

5.1 Abstract

This study investigated the effects on processing conditions including bran particle size, fermentation time, and baking time and temperature on the extractable antioxidant properties of whole-wheat pizza crust. Experiments were carried out using two different varieties of hard white winter wheat, Trego and Lakin, and antioxidant property assays included scavenging properties against peroxyl (ORAC), hydroxyl (HOSC), DPPH (RDSC), and cation ABTS radicals in addition to total phenolic contents (TPC) and ferulic acid compositions. Results indicated that increasing dough fermentation time from 0 to 48 hours had no significant influence on antioxidant properties except HOSC which increased as much 28%, possibly a result increases in soluble free ferulic acid which increased as much as 130%. Bran particle size had no effect on the antioxidant properties evaluated. Increasing baking temperature from 204 to 288 degrees C with a 7 minute bake time increased all evaluated antioxidant properties by as much as 82%. Increasing baking time from 7 to 14 minutes with 204 degrees C baking temperature increased some antioxidant properties as much as 60%, although some decreases were also observed. Results suggest that longer dough fermentation times, and increased baking time or temperature may be potential approaches to increase the antioxidant properties of

whole-wheat pizza crust.

5.2 Introduction

Increasing evidence has supported the role of whole-grain consumption in reducing the risk of chronic conditions such as cardiovascular disease and cancer (*1-4*). While the exact mechanism(s) responsible for these beneficial health effects have not been elucidated, it is thought that antioxidants present in whole-grains may play a role in this relationship (*2,5*). Antioxidants are thought to prevent chronic conditions by preventing oxidative damage to biomolecules through mechanisms such as free radical scavenging, transition metal ion chelation, or antioxidative enzyme system stimulation (*6, 7*).

Wheat (*Triticum spp.*) is an important dietary staple globally and is one of the most commonly consumed grains, representing 71% of grain consumption in the U.S. in 2003 (*8*). Recent *in-vitro* studies have shown whole-grain wheat to exhibit significant antioxidant properties including free radical scavenging activities against hydroxyl, peroxyl, superoxide anion, DPPH, and cation ABTS radicals, chelating activities towards reactive Fe^{2+} metals ions, and prevention of low-density lipoprotein and DNA oxidation (*9-14*). Phenolic acids, predominately ferulic acid, have been reported in significant levels in whole-wheat grains concentrated in the bran fraction, and are thought to be a major contributor to wheat antioxidant properties (*9, 12, 15-20*). Recent studies showing the antioxidant rich bran fraction of wheat, as opposed to other fractions, being the key factor responsible for reducing the risk of cancer and

cardiovascular disease observed in wheat grain support the role that whole-wheat antioxidants may play in health promotion (*3, 21, 22*).

Food processing induces complex physicochemical changes in food systems and has the potential to affect their antioxidant properties (*23, 24*). Potential mechanisms through which processing may effect the antioxidant properties of antioxidant compounds present in food can include chemical or enzymatic reactions such as oxidation or isomerization, leaching, or their release from or binding to food matrices (*23, 24*). Processing steps commonly utilized for whole-grain food production can include grain milling, mixing or shearing, kneading, fermentation, proofing, and thermal processing. While several previous studies on refined wheat products have shown processing steps including baking, fermentation, and kneading to induce significant losses of antioxidative carotenoids and tocopherols, no studies to date have investigated effects of these steps on antioxidant properties for whole-wheat food systems (*25-29*). The objective of this study therefore was to evaluate the effects of processing on whole-wheat food system antioxidant properties and phenolic acid contents, using a whole-wheat pizza crust food model. The effects of the following processing parameters were investigated in this study: bran particle size, dough fermentation time, and baking temperature and time.

5.3 Materials and Methods

Chemicals and Reagents. 2,2'-bipyridyl, 2,2-diphenyl-1-picryhydrazyl radical ($DPPH^{\bullet}$), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium

salt (ABTS), fluorescein (FL), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), and $FeCl_3$ were purchased from Sigma-Aldrich (St. Louis, MO). 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.

Hard Winter Wheat Samples and Preparation. Bran from Trego and Lakin wheat varieties, both commonly produced hard white winter wheat varieties, were provided by Dr. Scott Haley in the Department of Soil and Crop Science, Colorado State University, Fort Collins, CO 80523. Both varieties were grown in Fort Collins, Colorado during the 2004 growing season under agronomic practices considered typical for wheat production in eastern Colorado. Harvested grain samples from each location were cleaned using seed cleaners to remove all non-grain debris present and stored under ambient conditions. Grain samples were ground and fractions separated using a Brabender Quadromat Junior experimental mill. Bran fractions were further ground to 20 and 80-mesh using a coffee grinder. Fractions were recombined to create two whole-grain wheat flours with different bran mesh sizes for each wheat variety.

Pizza Crust Preparation. Pizza doughs were prepared by combining whole-wheat flour preparations, water, honey, soybean oil, dry active yeast (Fleischmann's, Fenton, Missouri), and salt at w/w percentages of 52.04, 33.24, 10.30, 2.34, 1.13, and 0.95 respectively using a KitchenAid stand mixer with dough hook on low speed for

1 minutes, followed by 3 minutes of kneading on low speed at ambient temperature. The dough was manually divided into 250 g pieces, rounded into boules, and allowed to ferment at 4 °C in covered stainless steel containers. Fermentation times tested included 0 (control), 18, and 48 hrs. Following fermentation, dough was tempered for 2 hrs at ambient temperature and rolled by hand into 12 inch crusts. Rolled crusts were transferred to aluminum pizza screens and baked in a conventional oven. Baking time-temperature combinations tested included 7 minutes at 204 °C, 14 minutes at 204 °C, and 7 minutes at 288 °F. For bran mesh size experiments, crusts were baked for 7 minutes at 204 °C. Following baking, pizza crusts were cooled for 30 min. at ambient temperature. After cooling or immediately after rolling for controls (unfermented dough), samples were frozen and freeze dried, and ground to 40-mesh.

Sample Extraction Procedure. One gram samples of 40-mesh freeze dried pizza crusts or doughs were extracted with 10 mL 50% acetone for 18 hours under nitrogen in the dark at ambient temperatures. The acetone extracts were used for $ABTS^{\bullet+}$ scavenging ability, oxygen radical absorbing capacity (ORAC), total phenolic contents (TPC) assay, hydroxyl radical scavenging capacity assay (HOSC), and relative $DPPH^{\bullet}$ scavenging capacity (RDSC) assays. Extracts were stored under nitrogen in the dark at ambient temperatures until further analysis.

Oxygen Radical Absorbing Capacity (ORAC) Assay. ORAC assay was conducted with fluorescein (FL) as the fluorescent probe using a Victor^3 multilabel

plate reader (PerkinElmer, Turku, Finland) according to a previous laboratory protocol (*9*) with modifications. Standards were prepared in 50% acetone 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 antioxidant extract, standard, or 100% ethanol for blanks, and 25 μL of 0.36 M AAPH. FL and antioxidant extracts were mixed in 96 well plate and pre-heated in plate reader for 20 minutes at 37 ºC after which the AAPH solution was added to initiate the antioxidant-radical reactions. The fluorescence of the assay mixture was recorded every minute for 80 minutes at 37 ºC. Excitation and emission wavelengths were 485 nm and 535 nm respectively. Results were expressed as μmoles of trolox equivalents (TE) per g sample (pizza crust or dough) on a dry weight basis.

Radical Cation $ABTS^{\bullet+}$ Scavenging Capacity. The free radical scavenging capacity of the 50% acetone extracts were evaluated against $ABTS^{\bullet+}$ generated according to a previously reported protocol (*30*). Fifty μL of the bran extracts were diluted to 500 μL with 100% ethanol to create working sample solutions. ABTS cation radicals were generated by oxidizing a 5 mM aqueous solution of ABTS with manganese dioxide for 30 min at ambient temperature. The final reaction mixture contained 80 μL of working sample solution or 100% ethanol for control, and 1.0 mL $ABTS^{\bullet+}$ solution with an absorbance of 0.7 at 734 nm. The absorbance at 734 nm was measured after a reaction time of 1 minute. Trolox equivalents (TE) were calculated using a standard curve prepared with trolox and expressed in μmoles TE per gram of sample (pizza crust or dough) on a dry weight basis.

Relative DPPH$^{\bullet}$ Scavenging Capacity (RDSC). The RDSC of the bran antioxidant extracts was determined following a recently reported procedure by Cheng and others (*31*). Briefly, 100 μL of antioxidant extract or 50% acetone for blank was added to 100 μL of freshly prepared DPPH$^{\bullet}$ solution to initiate antioxidant-radical reaction. The absorbance of the reaction mixtures were measured at 515 nm at 40 minutes of reaction. The initial concentration was of DPPH$^{\bullet}$ was 100 μM for all reaction mixtures. Results were calculated using areas under the curve relative to trolox standards. Results were expressed as micromoles trolox equivalents (TE) per gram sample (pizza crust or dough) on a dry weight basis.

Hydroxyl Radical Scavenging Capacity (HOSC) Assay. HOSC assay was conducted with 50% acetone solutions according to a previously published protocol (*10*) using a Victor^3 multilabel plate reader (PerkinElmer, Turku, Finland). Reaction mixtures consisted of 170 μL of 9.28×10^{-8} M FL prepared in 75 mM sodium phosphate buffer, 30 μL of standard or sample or blank, 40 μL of 0.1990 M H_2O_2, and 60 μL of 3.43 mM $FeCl_3$. Fluorescence was measured every minute for 3 hours with an excitation wavelength of 485 nm and emission wavelength of 535 nm. Trolox prepared in 50% acetone at concentrations of 20, 40, 60, 80, and 100 μM were used to prepare the standard curve for HOSC quantification. HOSC results were expressed as micromoles trolox equivalents (TE) per gram of sample (pizza crust or dough) on a dry weight basis.

Total Phenolic Contents. The 50% acetone extracts were analyzed for total

phenolic contents using the Folin-Ciocalteu reagent according to a previously reported procedure (*13*). Folin-Ciocalteu reagent was prepared by refluxing 85% phosphoric acid, sodium molybdate, sodium tungstate, and concentrated hydrochloric acid for 10 h, reacting with lithium sulfate, then oxidizing with bromine followed by filtration. The final reaction mixture contained 50 μL of antioxidant extracts, 250 μL freshly prepared Folin-Ciocalteu reagent, 750 μL 20% sodium carbonate, and 3 mL of ultra-pure water. Absorbance at 765 nm was read after a reaction time of 2 h at ambient temperature. Total phenolic contents calculated using gallic acid as a standard. TPC results were expressed as milligrams gallic acid equivalents (GAE) per gram of sample (pizza crust or dough) on a dry weight basis.

Phenolic Acid Composition. Pizza crust and dough samples were analyzed for their soluble free, soluble conjugated, insoluble bound, and total phenolic acid compositions using a previously reported procedure (*9*). Acetone/methanol/water (7/7/6, v/v/v) was used to extract the soluble free and the soluble conjugated phenolic acids, while the insoluble bound phenolic acids remained in the resulting solid residue. The free and conjugated phenolic acids in the acetone/methanol/water solution were separated based on their solubility under acidic condition (pH 2) by extracting soluble free phenolic acids into ethyl acetate and ethyl ether (1:1, v/v). Soluble conjugated phenolic acids were also hydrolyzed using NaOH, and re-extracted in ethyl acetate/ethyl ether (1:1, v/v) after the reaction pH was brought to pH 2. The solid residue with insoluble bound phenolic acids was hydrolyzed with NaOH, and the supernatant was re-extracted with ethyl acetate/ethyl ether (1:1, v/v)

after pH was adjusted to about pH 2. The concentration of NaOH in the hydrolysis reaction mixtures was 2 M. After evaporation of ethyl acetate and ethyl ether, each phenolic acid extract was quantitatively re-dissolved in MeOH and analyzed by HPLC using a Phenomenex C18 column (250 mm × 4.6 mm) according to an established protocol (*11*). 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). Solvent gradient was programmed from 10 to 100% B in 42 min with a flow rate of 1.0 mL/min (*11, 16*). Identification of phenolic acids was accomplished by comparing the retention time of peaks in the samples to that of the standards under the same HPLC conditions. Quantification of each phenolic acid was determined using external standards and total area under each peak.

Moisture Content. The moisture content of bran samples before and after the solid-state enzymatic reactions were determined using an oven following the AACC method 44-16 (*32*).

Statistical analysis. All treatments were conducted in triplicate. Data were reported as mean ± SD for triplicate treatments. ANOVA and Tukey's tests performed (SPSS for Windows, Version Rel. 10.0.5., 1999, SPSS Inc., Chicago, IL) to identify differences among means. Correlation analyses were performed using a two-tailed Pearson's correlation test. Statistical significance was declared at $P \leq 0.05$.

5.4 Results and Discussion

Effects of Bran Mesh Size on the Antioxidant Properties of Whole-Wheat Pizza Crust.

To evaluate the effects of bran particle size on extractable pizza crust antioxidant properties, pizza crusts were prepared using two bran particle sizes, 20- and 80-mesh, for two varieties of hard white wheat, and 50% acetone extracts analyzed. Results presented in **Table 5.1** indicate for both wheat varieties that decreasing the particle size from 20- to 80-mesh did not significantly alter antioxidant activities of pizza crust extracts. In addition, no significant differences between treatments for the two wheat varieties were observed, indicating that these two varieties of wheat have similar contributions to the antioxidant properties in baked pizza crusts. Results for $ABTS^{\bullet+}$ scavenging capacity ranged from 16.50 to 18.46 μmoles TE/g pizza crust on a dry weight basis, similar to results by Moore and others (*9*) for soft-wheat grains. ORAC ranged from 16.12 to 21.16 μmoles TE/g pizza crust on a dry weight basis, higher than results reported by Miller and others (*33*) for whole-wheat bread, but lower than results for soft wheat grains from Moore and others (*9*). The HOSC results ranged from 2.54 to 2.94 μmoles TE/g pizza crust on a dry weight basis, lower than that reported by Moore and others (*10*) for soft wheat grain. $DPPH^{\bullet}$ scavenging capacity expressed relative to trolox using the newly developed RDSC assay ranged from 1.60 to 1.93 μmoles TE/g pizza crust on a dry weight basis, similar to results reported by Cheng and others (*31*) for hard wheat grain. Total phenolic contents for samples ranged from 1.01 to 1.22 mg GAE/g pizza

crust on a dry weight basis, similar to that of whole-wheat bread reported by Gelinas and others (*34*).

Decreasing the particle size of bran is of interest to whole-wheat food producers, as it has been reported to help offset losses in loaf volume common in whole-wheat products as a result of added bran (*35, 36*). A study by Zhou and others (*15*) indicated that micronization of wheat bran increased its extractable antioxidant properties, but it was unclear from this study if these increases were a result of increased extraction surface area, or other processes involved in micronization such as thermal treatment. Another recent study by Cheng and others (*37*) found that while decreasing bran particle size may increase its extractable antioxidant properties, it also accelerates the loss of its antioxidants during storage and thermal processing. Results from the present study indicate that decreasing wheat bran particle size in whole-wheat pizza crust may not significantly impact its antioxidant properties. It is likely therefore, that decreasing bran particle size in whole-wheat food formulations to improve its physical quality attributes such as loaf volume, may not have undesirable effects on antioxidant properties in the finished food products.

Table 5.1. Effect of Bran Mesh Size on Pizza Crust Antioxidant Properties for Two Hard Wheat Varieties*

Wheat Variety	Mesh Size	ABTS (µmol TE/g pizza crust dw)	ORAC (µmol TE/g pizza crust dw)	HOSC (µmol TE/g pizza crust dw)	RDSC (µmol TE/g pizza crust dw)	TPC (mg GAE/g pizza crust dw)
Lakin	20	17.63 ± 0.17a	16.12 ± 1.2a	2.54 ± 0.38a	1.84 ± 0.26a	1.01 ± 0.09a
Lakin	80	18.37± 0.25a	17.87 ± 0.14a	2.94 ± 0.41a	1.93 ± 0.05a	1.14 ± 0.06a
Trego	20	16.50 ± 0.80a	19.67 ± 1.6a	2.70 ± 0.29a	1.71 ± 0.8a	1.16 ± 0.17a
Trego	80	18.46 ± 1.50a	21.16 ± 3.51a	2.72 ± 0.21a	1.60 ± 0.10a	1.22 ± 0.18a

*Pizza crusts baked at 204 °C for 7 minutes. TE stands for trolox equivalents; GAE stands for gallic acid equivalents; dw stands for dry weight basis; ABTS stands for $ABTS^{\bullet+}$ scavenging capacity; ORAC stands for oxygen radical absorbing capacity; HOSC stands for hydroxyl radical scavenging capacity; RDSC stands for relative $DPPH^{\bullet}$ scavenging capacity; TPC stands for total phenolic contents. Reported values are mean of triplicate treatments ± SD. Values marked by the same letter are not significantly different ($P < 0.05$). All results are expressed on a per dry pizza dough weight basis.

Effects of Dough Fermentation Time on the Antioxidant Properties of Whole-Wheat Pizza Crust

The fermentation of dough during the production of yeast leavened wheat products such as bread and pizza crust play an important role in the physicochemical and sensory properties of these foods. Yeast fermentation not only produces dough leavening CO_2 and ethanol which improves the physicochemical properties of flour proteins, but also numerous flavor compounds (*38*). Pizza crust preparation in particular often utilizes extended time, low temperature dough fermentations to improve its flavor properties (*39*). It is therefore of interest to understand how pizza dough fermentation may influence its antioxidant properties.

The effect of fermentation time on the antioxidant properties of whole-wheat pizza dough was evaluated by allowing dough samples to ferment for 0, 18, and 48 hours under refrigerated conditions at 4 ºC, and analyzing 50% acetone extracts of these samples. Results presented in **Table 5.2** for $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging capacity, ORAC, and TPC indicate that fermentation had no significant affect on these antioxidant properties for pizza dough extracts. HOSC results (**Figure 5.1**) for Lakin pizza dough, however, showed that 18 hours of fermentation compared to no fermentation (0 hr) caused a 25% increase in HOSC on a per dry pizza crust weight basis, while an additional 30 hours of fermentation (48 hr versus 18 hr fermentation) decreased HOSC by 44%. Trego pizza dough extracts, in contrast, showed no significant changes in HOSC from 0 to 18 hours and a significant 27% increase from

0 to 48 hours (**Figure 5.1)**. These HOSC results indicate that changes in pizza dough extract hydroxyl radical scavenging properties as a result of fermentation may be influenced by wheat variety. These results also indicate that the antioxidant capacity assay used may influence estimation of pizza dough antioxidant activity changes as a result of fermentation.

Table 5.2. Effect of Fermentation Time on Pizza Dough Antioxidant Properties for Two Hard Wheat Varieties.

Wheat Variety	Fermentation Time (hr)	ABTS (μmol TE/g pizza dough dw)	ORAC (μmol TE/g pizza dough dw)	RDSC (μmol TE/g pizza dough dw)	TPC (mg GAE/g pizza dough dw)
Lakin	0	17.9 ± 0.85a	26.26 ± 2.90a	1.72 ± 0.00a	1.53 ± 0.19a
Lakin	18	17.75 ± 0.09a	25.83 ± 3.10a	1.78 ± 0.19a	1.51 ± 0.24a
Lakin	48	17.16 ± 0.02a	25.69 ± 2.51a	1.52 ± 0.07a	1.50 ± 0.14a
Trego	0	17.79 ± 0.57a	28.23 ± 3.04a	1.51 ± 0.17a	1.61 ± 0.06a
Trego	18	18.35 ± 0.27a	29.82 ± 3.45a	1.51 ± 0.11a	1.52 ± 0.04a
Trego	48	18.24 ± 0.17a	29.31 ± 0.07a	1.54 ± 0.13a	1.56 ± 0.15a

Dough fermentations were carried out at 4 ºC. TE stands for trolox equivalents; GAE stands for gallic acid equivalents; dw stands for dry weight basis; ABTS stands for $ABTS^{\bullet+}$ scavenging capacity; ORAC stands for oxygen radical absorbing capacity; RDSC stands for relative $DPPH^{\bullet}$ scavenging capacity; TPC stands for total phenolic contents. Reported values are mean of triplicate treatments ± SD. Values marked by the same letter are not significantly different ($P < 0.05$). All results are expressed on a per dry pizza dough weight basis.

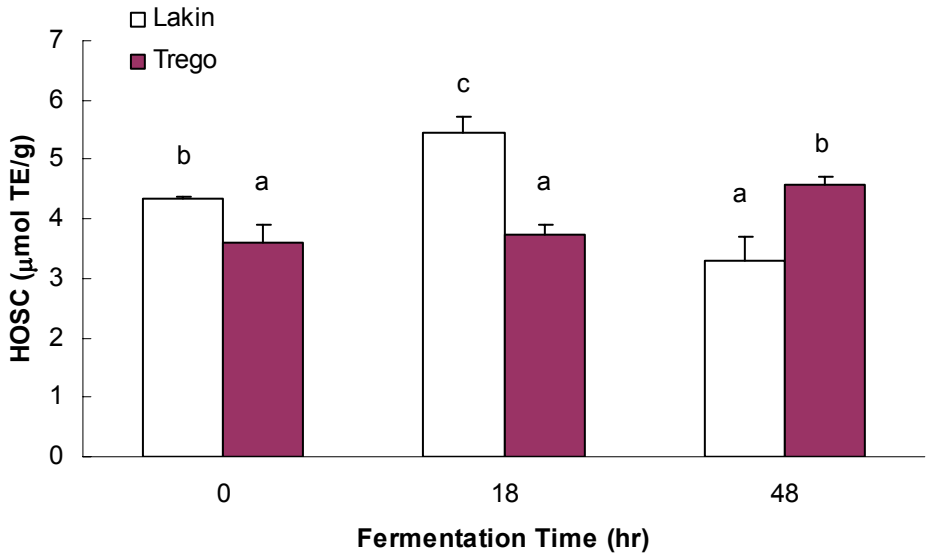

Figure 5.1. Effects fermentation time on the hydroxyl radical scavenging capacities (HOSC) of pizza dough for two hard wheat varieties. Dough fermentations were carried out at 4 ºC. Results expressed as µmoles trolox equivalents per gram of pizza dough on a dry weight basis. All treatments were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

Figures 5.2-5.4 show changes in soluble free, soluble conjugated, and insoluble bound ferulic acid contents, respectively, on a per dry pizza crust weight basis during fermentation for Trego and Lakin pizza dough. The soluble free ferulic acid contents of both wheat varieties increased significantly as result of fermentation, and Trego pizza dough showed a significant time-dependent increase (**Figure 5.2**). Trego and Lakin pizza doughs fermented 48 hours showed significant 130% and 75% increases, respectively, in soluble free ferulic acid contents compared to no fermentation. Soluble conjugated ferulic acid contents of Trego pizza dough showed a slight, but not significant, decrease after 18 hours while Lakin showed no changes (**Figure 5.3**). The insoluble bound ferulic acid contents of Lakin pizza dough showed a significant 61% decrease as a result of 48 hrs fermentation compared to no

fermentation, while Trego pizza dough showed no significant changes as a result fermentation (**Figure 5.4**). Comparing ferulic acid composition changes between Trego and Lakin pizza doughs, fermentation significantly increased soluble free ferulic acid for both varieties, and decreased either soluble conjugated or insoluble bound ferulic acid. Two previous studies on rye sourdough bread have also shown a significant increase in soluble free phenolic acids as a result of dough fermentation (*40, 41*).

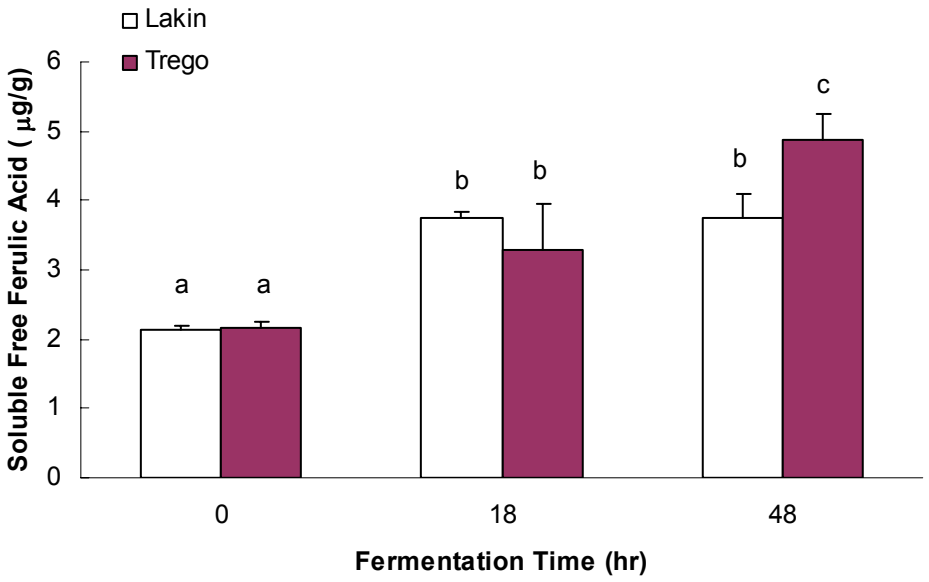

Figure 5.2. Effects fermentation time on the soluble free ferulic acid contents of pizza dough for two hard wheat varieties. Dough fermentations were carried out at 4 ºC. Results expressed µg ferulic acid per gram of pizza dough on a dry weight basis. All treatments were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

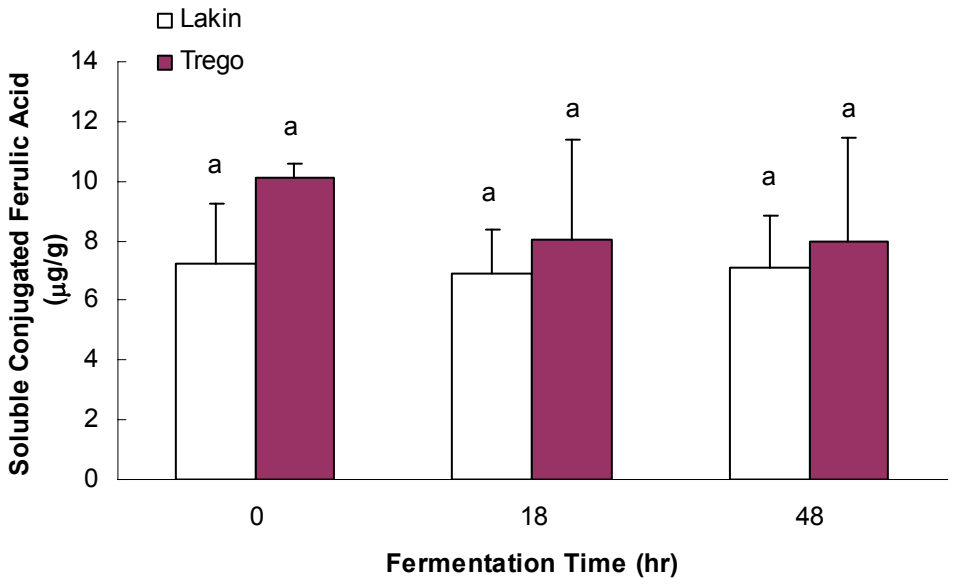

Figure 5.3. Effects fermentation time on the soluble conjugated ferulic acid contents of pizza dough for two hard wheat varieties. Dough fermentations were carried out at 4 ºC. Results expressed µg ferulic acid per gram of pizza dough on a dry weight basis. All treatments were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

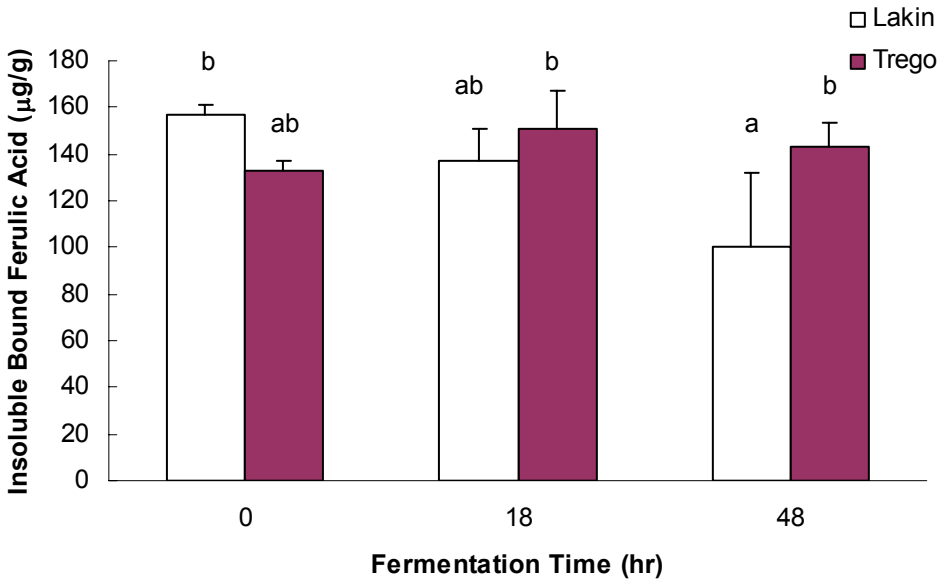

Figure 5.4. Effects fermentation time on the insoluble bound ferulic acid contents of pizza dough for two hard wheat varieties. Dough fermentations were carried out at 4 ºC. Results expressed µg ferulic acid per gram of pizza dough on a dry weight basis. All treatments were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

The changes in ferulic acid composition during dough fermentation observed in this study could potentially be a result of enzymatic hydrolysis of insoluble bound or soluble conjugated ferulic acid by enzymes produced from yeast or other microorganisms and enzymes present in the dough. Recent studies have found enzyme preparations with xylanase and feruloyl esterase activities capable of hydrolyzing insoluble bound ferulic from wheat bran (*30, 42*). Another recent study by Moore and others (*43*) found solid-state yeast fermentations of wheat bran to increase soluble free ferulic acid contents, possibly as a result of hydrolytic enzymes produced by the yeasts tested. More studies are necessary, however, to determine the exact biochemical mechanisms for these changes in phenolic acid compositions during whole-wheat pizza dough fermentation.

Effects of Baking Time and Temperature on the Antioxidant Properties of Whole-Wheat Pizza Crust

Thermal processes such as baking can induce physicochemical changes in whole-grain based products capable of altering their antioxidative chemical compositions such as carotenoid and tocopherol contents (*44*). It is of interest therefore how baking conditions may alter the antioxidant properties of whole-wheat pizza crust. This study compared the effects three baking time/temperature combinations, 7 minutes at 204 °C, 14 minutes at 204 °C, and 7 minutes at 288 °C, on the antioxidant properties of whole-wheat pizza crust extracts for two varieties of whole-wheat flour. Unbaked dough results were included as the control to understand how the baking step itself alters the antioxidant properties of whole-wheat

pizza dough.

Results presented in **Figures 5.5-5.9** showed some significant differences between baked and unbaked pizza dough extracts, and between the tested baking conditions for some antioxidant capacity assays, with results expressed on a per dry pizza crust weight basis. Results presented in **Figure 5.5** for $ABTS^{\bullet+}$ scavenging capacity, indicate that increasing thermal treatment from 7 to 14 min at 204 °C or from 204 to 288 °C at 7 min significantly increases $ABTS^{\bullet+}$ scavenging properties between 42 and 47% for both wheat varieties. Compared to the unbaked dough (control), baking at 204 °C for 7 minutes did not significantly alter extract $ABTS^{\bullet+}$ scavenging properties, while both more intense baking conditions did significantly increase them. Results for relative $DPPH^{\bullet}$ scavenging capacity (RDSC) in **Figure 5.6** indicate that increasing thermal treatment from 7 to 14 min at 204 °C or from 204 to 288 °C at 7 min significantly increased RDSC between 50 and 82% for pizza crust extracts from both wheat varieties. Compared to un-baked dough (control) extracts, baking at 204 °C for 7 minutes did not significantly alter $ABTS^{\bullet+}$ scavenging properties, while both more intense baking conditions did significantly increase them. RDSC and $ABTS^{\bullet+}$ scavenging property results were found to be highly correlated under experimental conditions using Pearson's correlation analysis ($r = 0.923$, $P < 0.01$, $n = 24$).

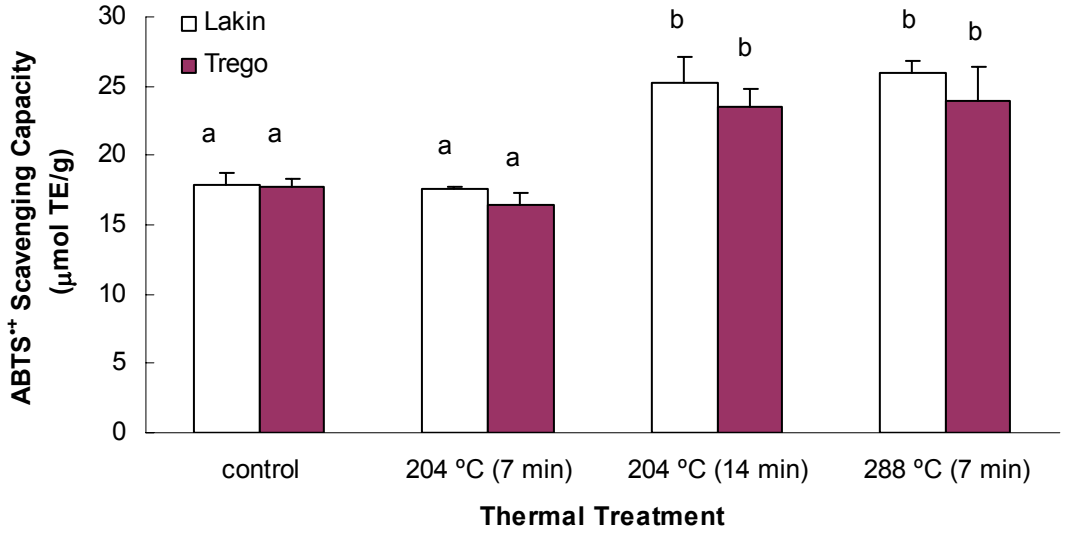

Figure 5.5. Effects of baking time and temperature on the ABTS$^{\bullet+}$ scavenging capacities of pizza crusts for two hard wheat varieties. Controls were dough samples not baked. Treatment samples were baked in a conventional oven. Results expressed as µmoles trolox equivalents per gram of sample (dough or baked crust) on a dry weight basis. All treatments were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

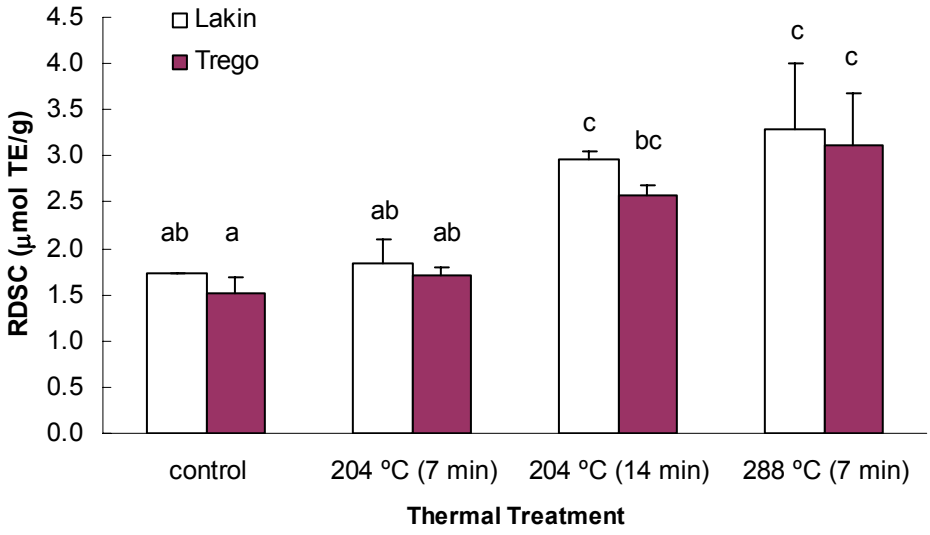

Figure 5.6. Effects of baking time and temperature on the relative DPPH$^{\bullet}$ scavenging capacities (RDSC) of pizza crusts for two hard wheat varieties. Controls were dough samples not baked. Treatment samples were baked in a conventional oven. Results expressed as µmoles trolox equivalents per gram of sample (dough or baked crust) on a dry weight basis. All treatments were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

Oxygen radical absorbing capacity (ORAC) results (**Figure 5.7**) showed no significant differences between pizza crusts extracts baked at 204 °C for 7 or 14 minutes, while changing baking conditions from 204 °C with 14 minutes to 288 °C with 7 minutes significantly increased ORAC between 47 and 51% for the two wheat varieties. In contrast to ABTS and RDSC results, baking at 204 °C for 7 or 14 minutes resulted in a significant decrease in ORAC compared to un-baked dough (control), while 7 minutes at 288 °C ORAC results were not significantly different than controls. Results for hydroxyl radical scavenging capacity (HOSC) shown in **Figure 5.8** indicate, similar to ORAC, that baking 204 °C for 7 or 14 minutes significantly decreased the HOSC of pizza crust extracts compared to dough (control) extracts. Baking at 288 °C for 7 minutes showed an increase in HOSC compared to both treatments at 204 °C, but were only significant for the Trego wheat variety. ORAC results were highly correlated to HOSC results under experimental conditions ($r = 0.738$, $P < 0.01$, $n = 24$).

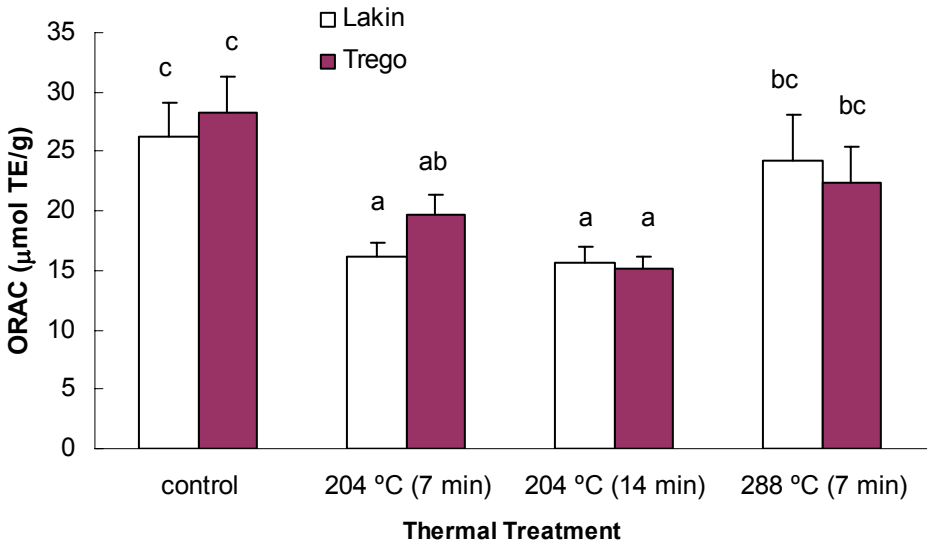

Figure 5.7. Effects of baking time and temperature on the oxygen radical absorbing capacities (ORAC) of pizza crusts for two hard wheat varieties. Controls were dough samples not baked. Treatment samples were baked in a conventional oven. Results expressed as µmoles trolox equivalents per gram of sample (dough or baked crust) on a dry weight basis. All treatments were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

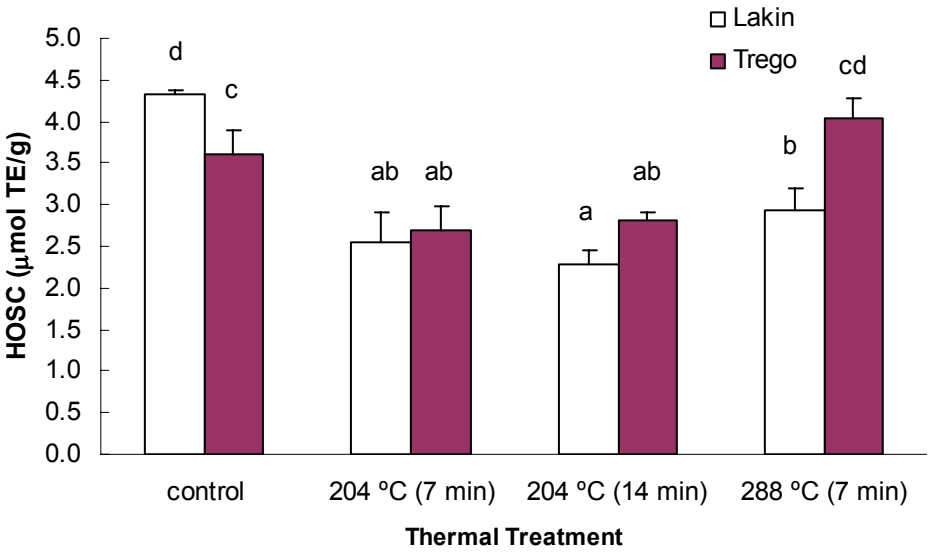

Figure 5.8. Effects of baking time and temperature on the hydroxyl radical scavenging capacities (HOSC) of pizza crusts for two hard wheat varieties. Controls were dough samples not baked. Treatment samples were baked in a conventional oven. Results expressed as µmoles trolox equivalents per gram of sample (dough or baked crust) on a dry weight basis. All treatments were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

The total phenolic contents (TPC) for pizza crust extracts were estimated using the Folin-Ciocalteu reagent, with results shown in **Figure 5.9**. Of the three baking conditions evaluated, the 288 °C, 7 minute baking conditions showed the highest TPC for both wheat varieties, but few significant differences were found between baking conditions. Similarly, few significant differences were found between un-baked and baked extracts. TPC results for baking extracts were highly correlated with all other antioxidant capacity results, RDSC ($r = 0.476$, $P < 0.05$, $n = 24$), $ABTS^{\bullet+}$ scavenging capacity ($r = 0.429$, $P < 0.05$, $n = 24$), HOSC ($r = 0.601$, $P < 0.01$, $n = 24$), and ORAC ($r = 0.711$, $P < 0.01$, $n = 24$).

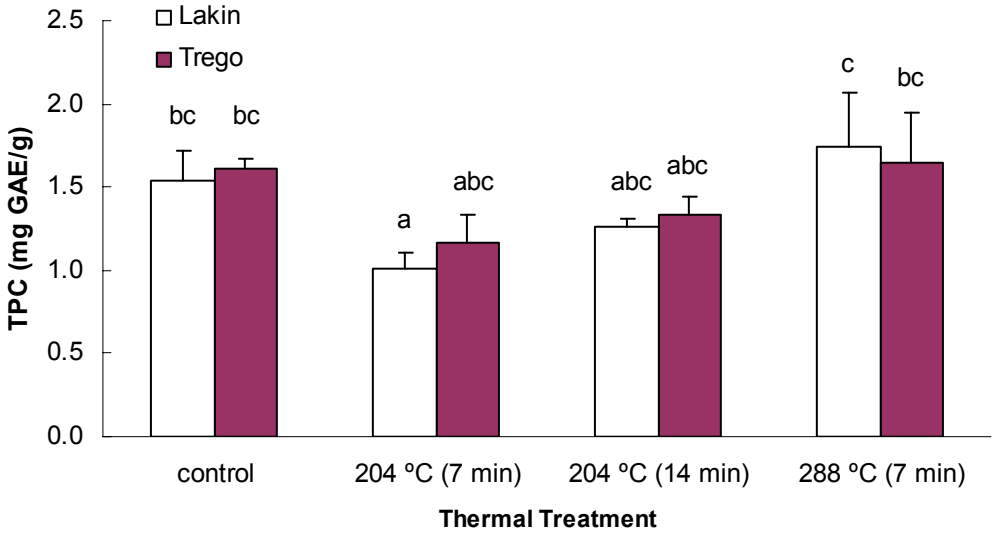

Figure 5.9. Effects of baking time and temperature on the total phenolic content (TPC) of pizza crusts for two hard wheat varieties. Controls were dough samples not baked. Treatment samples were baked in a conventional oven. Results expressed mg gallic acid equivalents (GAE) per gram of sample (dough or baked crust) on a dry weight basis. All treatments were conducted in triplicate, and mean values are reported. The vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ($P < 0.05$).

Overall results comparing baked with un-baked (control) samples under

experimental conditions indicate that baking whole-wheat pizza dough has the potential to significantly alter its antioxidant capacity dependent on antioxidant capacity assay utilized. Comparing the effects of baking conditions, results from this study indicate that increasing either baking temperature or time for whole-wheat pizza crust has the potential to increase its antioxidant properties, depending on the antioxidant capacity assay used. The most statistically significant increases in whole-wheat pizza crust antioxidant properties, however, were observed when increasing baking temperature from 204 to 288 °C at 7 minutes of baking time. The two hard wheat varieties used in this study showed similar effects from baking conditions for each antioxidant property measured, indicating that baking conditions as opposed to wheat variety may be more significantly influence how baking treatments effect whole-wheat pizza crust antioxidant properties. Results from this study also demonstrate that the antioxidant capacity assay utilized can significantly alter the estimation of antioxidant property changes in pizza crust as a result of baking conditions.

While no previous studies have evaluated the effects of baking on pizza crust antioxidant properties, four studies have investigated this effect for whole-grain breads. Two studies found slight but not statistically significant increases in TPC, soluble free ferulic acid, or DPPH radical scavenging capacity for extracts of baked whole-grain rye sourdough bread versus fermented dough (*40, 41*). Leenhardt and others (*44*) found baked whole-grain rye sourdough bread extracts to have significantly lower levels of carotenoids and tocopherols compared to fermented

dough, but did not investigate other antioxidant properties. Another study by Lindenmeier and others (*45*) found both increased baking time and temperature to increase antioxidant activity for sourdough rye bread crust extracts measured using an inhibition of linoleic acid peroxidation assay, but found the opposite trend for crumb extracts from the same bread samples. This study by Lindenmeier attributed the increases in crust extractable antioxidant properties from increased thermal treatments to Maillard reaction products, particularly 2,4-dihydroxy-2,5-dimethyl-1-(5-acetamino-5-methoxycarbonyl-pentyl)-3-oxo-2H-pyrrol, which was found to significantly increase in bread crust samples baked for longer times or temperatures (*45*).

To better understand the mechanisms for results observed in this study as a result of baking conditions for whole-wheat pizza crust, further studies are necessary. These should include investigating changes in phenolic acid compositions, as well as Maillard reaction products.

5.5 Conclusion

In summary, the present study demonstrates for the first time that changes in processing steps including dough fermentation time and baking conditions have the potential to increase whole-wheat pizza crust antioxidant properties, while changes in bran particle size are not likely to affect these antioxidant properties. Further studies are necessary to understand mechanisms for these changes, and to optimize processing procedures for improved antioxidant properties.

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