Wheat antioxidants: understanding changes of phenolic profiles in the wheat food chain and developing rapid quantification methods

by

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B.S., Shandong University, 2014
M.A., University of Delaware, 2016

AN ABSTRACT OF A DISSERTATION

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Abstract

The health benefits of whole wheat foods can partially be attributed to the phytochemicals. In recent years, driven by humanity' desire for healthier food sources, potential health benefits of wheat grains have become significant quality parameters. The goal of this study is to understand the changes in phenolic acid profiles and antioxidant potential in the wheat food chain and develop novel spectroscopy methods for rapid quantification of total phenolics in whole wheat. Specific objectives are to: 1) understand the effect of wheat varieties, environment, fertilizer, integrated managements, and their interactions on wheat phenolics; 2) investigate the effect of grain and food processing such as germination, fermentation, and baking; 3) examine potential bioaccessibility of wheat phenolics in the human digestive tract and release of phenolics by gut microbiota fermentation; and 4) develop models based on UV-Vis and NIR spectroscopies and chemometrics for rapid quantification of total phenolic content (TPC) in wheat.

The study on phenolic profiles of 12 Kansas winter wheat varieties showed that most wheat phenolics (> 90%) existed in insoluble-bound forms, and phenolic content and composition were highly dependent on the varieties. The study on year, variety, and fertilizer effect indicated that the year effect was significant for TPC and most phenolic acids. Increased nitrogen application led to increased production of *trans*-ferulic acid, and sulfur application affected the response to nitrogen application. Varieties also differed in the response of phenolic acid concentration and composition to sulfur application. Results also showed that year x location x management and year x management x variety interactions were significant for TPC. Year x location x variety x management was significant for most phenolic acids. Managements

with no fungicide application may lead to increased accumulation of phenolic compounds, especially for varieties that are more susceptible to the fungi infection.

Due to significant effect from varieties, different varieties were included in the studies of processing effects. Seed germination at early-stage (<24 h) did not influence baking properties of whole flours but decreased TPC, flavonoid content, antioxidant activities, and phenolic acid concentrations for three varieties. Bread-making steps including fermentation and baking had positive effects on phenolic antioxidants, especially for the soluble fractions. Some soluble phenolic acids were incorporated into Maillard reaction products. Simulated digestion released more soluble *trans*-ferulic acid than chemical extraction from bread (17.69 to 102.71 µg/g), cookie (15.81 to 54.43 µg/g), and pasta (4.88 to 28.39 µg/g). Colon fermentation further released phenolics from the food matrix. In general, phenolics in whole wheat can be well retained from farm to fork and exhibit considerable bioaccessibility in the human digestive tract.

Partial least square (PLS) models were successfully developed using UV-Vis spectra to predict TPC (R^2-calibration=0.89, and R^2-validation=0.89) and *trans*-ferulic acid (R^2-calibration=0.82, and R^2-validation=0.85) in wheat extracts. Moreover, a simpler NIR model that can directly predict the TPC (R^2-calibration=0.92, and R^2-validation=0.90) of whole wheat flour was also established. Breeding programs that require fast screening and selection of wheat lines with enriched phenolic compounds will greatly benefit from these new techniques.

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Dedication

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Preface

The Preface page is optional. If you include it, retain the Preface heading and enter your text here.

Chapter 1 - Literature review

1.1. Introduction

Whole grains are defined by the American Association of Cereal Chemists International and the FDA as products consisting of the "intact, ground, cracked or flaked fruit of the grain whose principal components, the starchy endosperm, germ and bran, are present in the same relative proportions as they exist in the intact grain (Jonnalagadda et al., 2011). In contrast, refined grains only retain the endosperm. The bran layer is mainly composed of non-digestible, insoluble carbohydrates such as cellulose and hemicelluloses. Epidemiological studies have found that consumption of whole wheat products may reduce risk of chronic diseases such as obesity, type 2 diabetes, cardiovascular diseases, and cancer (Jonnalagadda et al., 2011; Liu, 2007). The 2005 Dietary Guidelines for Americans recommends Americans to consume at least three ounce-equivalents of whole grain products each day (USDA, 2005). Wheat, rice, and corn are the major grains in the human diet. Other minor grains include oat, barley, rye, triticale, sorghum, millet, and buckwheat. Wheat (*Triticum spp.*) is a worldwide staple food (Godfray et al., 2010). World trade of wheat is greater than that for all other crops combined (Giraldo et al., 2019). Since rice is normally consumed after refining (though there is also brown rice available) and food products from corn are less popular in the western countries, whole wheat products might be the most important source for whole grain intake.

The health benefits of whole wheat products can be partially attributed to their phytochemical content and dietary fibers (Okarter & Liu, 2010). Phytochemical is generally used to describe plant compounds that are under research with unestablished effects on health and are not

scientifically defined as essential nutrients (Liu, 2004). Common phytochemicals in whole wheat is presented in **Figure 1-1**. Due to consumers' desires for healthy food ingredients and products, tremendous amount of studies have been reported on phytochemical profiles of whole wheat, their health-promoting effects, and underlying mechanisms(Moore et al., 2005). Wheat breeders and producers also consider the concentration of phytochemicals as another attribute in evaluating wheat quality in addition to conventional targets such as grain yield and end-use properties (Tian et al., 2020). Processing technologies have also been developed to increase nutraceutical values of whole wheat flour and whole wheat products. This chapter summarized current studies on 1) phytochemical profiles and antioxidant activities of whole wheat; 2) epidemiological studies on health benefits of whole wheat products; 3) effect of wheat genotypes and growing environments (including farming managements) on wheat bioactive compounds; and 4) effect of grain and food processing on wheat bioactive compounds.

1.2. Phytochemical profile and antioxidant activity

1.2.1. Phenolic acids

Phenolic acids are consisted of a phenolic ring and an organic carboxylic acid functional group. Phenolic acid is the most abundant phytochemical found in wheat (Okarter et al., 2010). There are two major groups of phenolic acids: derivatives of hydroxybenzoic acid and derivatives of hydroxycinnamic acids. Common phenolic acids found in whole wheat include 4-hydroxybenzoic acid, caffeic acid, vanillic acid, syringic acid, *para*-coumaric acid, *trans*-ferulic acid, sinapic acid, and *cis*-ferulic acid (**Table 1-1**). These phenolic acids generally exist in soluble-free, soluble-conjugated, and insoluble-bound forms (Adom et al., 2003). *Trans*-ferulic acid is the most predominant phenolic acid found in wheat (Tian & Li, 2018). Majority of ferulic

acids are bounded with oligosaccharides by esterification linkages (Ishii, 1997). Soluble-free phenolic acids can be extracted by organic solvents such as 80% ethanol or a mixture of methanol: acetone: water = 7:7:6 (v/v/v). Soluble-conjugated phenolic acids can be further released by alkaline hydrolysis from the soluble extraction. Insoluble-bound phenolic acids are those released by alkaline treatment of the residues after solvent extraction (Moore et al., 2005). HPLC-DAD or HPLC-MS system equipped with a C18 column are often used to separate and quantify different phenolic acids. Phenolic acid compositions of whole wheat have been extensively reported. A recent literature review reported that concentration of soluble-free, soluble-conjugated, and insoluble-bound *trans*-ferulic acid ranged from 0.6 to 39, 1.9 to 64.5, and 259.8 to 702.6 µg/g, respectively (Liu et al., 2020). Total concentration of *para*-coumaric acid ranged from 10.4 to 92.4 µg/g. Total concentration of syringic acid ranged from 7.6 to 29.5 µg/g. Though trans-ferulic acid is the most predominant phenolic acid in the insoluble fractions, a previous study found that sinapic acid was the most abundant for some wheat genotypes in the soluble fractions (Tian et al., 2019). Total concentration of vanillic acid ranged from 5.6 to 19.5 µg/g. Besides monomeric phenolic acids, di-ferulic acids (DFA), flavonoids, carotenoids and alkylresorcinols are also characterized by previous studies and summarized in **Table 1-2**. DFA isomers were also identified and quantified in some previous studies (Gong et al., 2019; Tian et al., 2021a). Common DFA isomers are 8-8 DFA, 8-5 DFA, 5-5 DFA, 8-O-4' DFA, and 8-5 benzofuran DFA. 8-O-4' DFA is reported to be the predominant form of DFA.

1.2.2. Flavonoids

Flavonoids have a 15-carbon skeleton consisting of two benzene rings and a heterocyclic ring (C_6-C_3-C_6 structure). Based on the different C_6-C_3-C_6 skeletons, flavonoids are divided into six

different classes: anthocyanidins, chalcones, flavones, flavonols, flavanones, and isoflavones (Panche et al., 2016). Flavonoids in wheat exist mainly in C-glycoside forms (Geng et al., 2016). The concentration of flavonoid compounds in whole wheat is lower compared to that in fruits and vegetables. Therefore, there are relatively fewer literatures on identifications of different flavonoid compounds in whole wheat. Anthocyanins from purple wheat have been analyzed in previous studies, which identified 13 anthocyanins and found that cyanidin 3-glucoside was the predominant anthocyanin in purple wheat (Hosseinian et al., 2008). Geng et al. (2016) identified 72 flavone C-glycosyl derivatives in wheat germs using UPLC-PDA-ESI/HRMS and mass defect filtering protocols.

Flavonoid contents of whole wheat are commonly determined by *in vitro* colorimetric assays and expressed as catechin equivalence (CE) per gram whole flour (Granato et al., 2018). Similar to phenolic acids, most flavonoid compounds exist in insoluble-bound fractions. Tian & Li (2018) determined flavonoid concentration of 12 hard red winter wheat varieties and found that soluble flavonoids ranged from 0.03 to 0.06 mg CE/g wheat, while insoluble flavonoids ranged from 0.33 to 0.73 mg CE/g wheat. Similarly, Leoncini et al. (2012) reported that total flavonoid contents of six wheat varieties ranged from 0.20 to 0.68 mg CE/g wheat.

1.2.3. Carotenoids

Over 750 kinds of carotenoids have been identified in nature (Nisar et al., 2015). In recent years, carotenoids have been extensively studied as provitamin-A and antioxidants in the human diet. Lutein, zeaxanthin, β -cryptoxanthin, β -carotene are common carotenoids found in whole wheat (Britton, 1995). Adom et al. (2003) analyzed carotenoids of 11 wheat varieties. Lutein

concentration ranged from 0.24 to 1.43 μg/g grain; zeaxanthin concentration ranged from 0.08 to 0.27 μg/g grain; β-cryptoxanthin concentration ranged from 0.01 to 0.13 μg/g grain. Moore et al. (2005) analyzed carotenoids of 12 soft wheat varieties and found that the concentration ranges for β-carotene, zeaxanthin, and lutein were 0.10−0.21, 0.20−0.39, and 0.82−1.14 μg/g of soft wheat grain, respectively. Okarter & Liu (2010) reported that lutein concentration ranged from 0.67 to 2.11 μg/g grain; zeaxanthin concentration ranged from 0.25 to 0.53 μg/g grain; β-cryptoxanthin concentration ranged from 0.12 to 0.20 μg/g. In general, some carotenoids compounds have been characterized in previous studies, but their concentrations are much lower compared to phenolic acids.

1.2.4. Alkylresorcinols

Alkylresorcinols (ARs) , also known as resorcinol lipids, are phenolic lipids composed of odd-numbered aliphatic chains and resorcinol-type phenolic rings (Kozubek & Tyman, 1999). ARs in wheat grain usually contained an aliphatic tail with 17, 19, 21, 23 or 25 carbons. ARs mostly exist in the wheat bran layers and absent in the refined flour (Tłuścik, 1978). Therefore, ARs are potential biomarkers for consumption of whole wheat. For this reason, ARs are attracting increasing research interests. A clinical study found that plasma AR concentrations were positively correlated with the amount of whole grains consumed (Ross et al., 2012). Ross et al. (2003) reported that the AR concentrations of 13 wheat genotypes ranged from 200 to 1489 μg/g bran. Liu et al. (2018) reported that concentration of ARs ranged from 697 to 1732 μg/g bran for 21 wheat varieties. These results suggest that wheat genotype has a significant effect on the concentration of ARs in wheat. Metabolites of ARs in biological sample have also been described in previous literatures (Ross et al., 2004; Wierzbicka et al., 2015).

1.2.5. *In vitro* antioxidant activity

According to the statement by Halliwell & Gutteridge, (2015), “an antioxidant is a substance that, when present at a low concentration compared with that of an oxidizable substrate in the medium, inhibits oxidation of the substrate”. Under this definition, wheat phytochemicals including phenolic acids, flavonoids, carotenoids, and others are considered as natural antioxidants. There are three major mechanisms of antioxidants: hydrogen atom transfer (HAT), single electron transfer (SET), and transition metal chelation. In HAT mechanism, an antioxidant (AH) quenches free radicals by donating hydrogen and stabilizing the peroxyl radical through resonance according to Eq. (1) (Brewer, 2011):

$AH + ROO^{\bullet} \rightarrow ROOH + A^{\bullet}$ (1)

In SET mechanism, antioxidant (AH) transfers one electron to reduce free radicals, pro-oxidant metals ions such as Fe^{2+} and Cu^{2+} (Apak et al., 2007).

$AH + ROO^{\bullet} \rightarrow ROO^{\bullet -} + AH^{+}$ (2)

$AH^{+} + H_2O \leftrightarrow A^{\bullet} + H_3O^{+}$ (3)

$ROO^{\bullet -} + H_3O^{+} \leftrightarrow ROOH + H_2O$ (4)

Examples of SET methods include cupric-ion reducing antioxidant capacity (CUPRAC), ferric reducing ability of plasma (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) free radical scavenging assays. Folin-Ciocalteu’s reagent is used to determined “total phenolic content” (TPC), however, reducing of the Folin- Ciocalteu’s reagent is also through SET mechanism. The most used HAT method is the oxygen radical absorbance capacity (ORAC) assay and inhibition of lipoperoxidation method (Shahidi & Zhong, 2015). These antioxidant assays are non-specific, and the results are usually

expressed using a standard such as gallic acid equivalence (GAE) for TPC and Trolox equivalence for ABST, DPPH, and ORAS assays. Moore et al. (2005) reported that TPC of 12 soft wheat varieties ranged from 0.40 to 0.80 mg GAE/g flour. Okarter et al. (2010) reported that TPC of 6 diverse wheat varieties ranged from 1.43 to 1.87 mg GAE/g flour. A study analyzing more samples by Tian et al. (2020) reported that TPC of 80 wheat varieties ranged from 1.40 to 2.45 mg GAE/g. Results of radical scavenging assays are usually expressed as µmol TE/g flour. Tian & Li (2018) reported that ABTS results of 12 hard red winter wheats ranged from 42.07 to 77.51 µmol TE/g flour. Moore et al. (2005) reported that ABTS of 12 soft wheat varieties ranged from to 14.3 to 17.6 µmol TE/g flour, and ORAC of these samples ranged from 32.9 to 47.7 µmol TE/g. Okarter et al. (2010) reported that ORAC of 6 wheat varieties ranged from 56.5 to 96.1 µmol TE/g. Results from different literatures show large variations regarding the antioxidant activity results. Wheat varieties, growing environments, and extraction methods can all influence the measurements. Therefore, results from different studies can sometimes be difficult to compare.

There are many criticisms on *in vitro* assays because free radicals in these assays such as ABTS and DPPH are not biologically relevant, and the mechanism of these reaction are of great complexity (Apak et al., 2013). Furthermore, there are no correlations between results from different assays (Moore et al., 2005). Due to these drawbacks, some studies recommend the discontinue of ABTS/DPPH assays and significant revision of ORAC assay (Schaich et al., 2015). However, there are still recent studies supporting the use of these methods for screening and quality control purposes (Barba et al., 2013; de Camargo et al., 2019). The usefulness of *in vitro* results cannot be ruled out. Though animal models or human studies are more appropriate,

these studies are expensive and time-consuming comparing to *in vitro* chemical methods. Some recent studies also reported associations between *in vitro* assays and *in vivo* measurements (Aouachria et al., 2017; Donado-Pestana et al., 2018; Villa-Hernández et al., 2017). In general, though *in vitro* assays (e.g., TPC, ABTS, DPPH, ORAC) have some limitations, they are still of great values especially for screening purposes.

1.3. Health benefits of whole grain consumption

1.3.1. Weight control

Epidemiological studies have found that consumption of whole grain foods help reduce risks of obesity and weight gain. It was showed that intakes of whole grains were also associated with lower BMI and lower abdominal fat (Esmaillzadeh et al., 2005; McKeown et al., 2002). Other studies found that intakes of whole grains help maintain smaller waist circumferences in adults (Jonnalagadda et al., 2011). Prospective epidemiological studies indicated that people with higher amount of whole grain intake are less likely to gain weight in a long term (Liu et al., 2003). An eight-year study showed that increased intake of whole grain by 40 grams per day lowered the eight-year weight gain by 1.1 kg (Koh-Banerjee et al., 2004).

Several mechanisms have been proposed to explain the role of whole grain diets in weight management. Firstly, dietary fibers in whole grain foods may influence food volume, energy density, gastric emptying, and glycemic responses (Schroeder et al., 2009). Whole grains may also play a role in promoting feeling of satiety and therefore lower the whole energy intake (Holt et al., 2001). Furthermore, recent studies found that phytochemicals from various food sources may inhibit the digestive enzyme activities (Fettach et al., 2019; Tan & Chang, 2017) which may

explain the low glycemic response and promoted satiety of whole grain consumption (Velickovic & Stanic-Vucinic, 2018).

1.3.2. Diabetes and inflammation

Balanced diet is a basic tool for the control of blood glucose levels (Gil et al., 2011). Several studies have confirmed that increased intake of whole grain foods has been associated with a 20–30 % reduction in the risk of type II diabetes. Montonen et al. (2003) found that there was an inverse correlation between whole-grain intake and the risk of type II diabetes whereas fibers from fruits and vegetables did not have this reduced effect on risk of type II diabetes. A long-term study of 90,000 women (Monro & Shaw, 2008), and another similar long-term study of 45,000 men (Riccardi et al., 2008), both indicated that those who consumed more cereal fibers had an approximately 30 % lower risk of developing type II diabetes, compared with those with lower consumption. In another study, individuals consumed mainly refined grains had a 57 % greater risk of type II diabetes than individuals with higher quantities of whole grain consumption (Henry et al., 2006). These benefits might be attributed to the dietary fiber contents of whole grains. Wholegrain cereals present lower glycemic index (GI) values compared to the refined grains. The incorporation of soluble dietary fibers in whole grain products increases viscosity of the bolus, lower the access of digestive enzymes, and limits the diffusion of the glucose, giving these products a far lower GI. In addition, organic acids in whole grain products may diminish postprandial glycaemia and insulinaemia effect. The role of ARs in reduced risk of type II diabetes has also been recognized. Through mice experiments, Oishi et al. (2015)found that wheat ARs increased glucose tolerance and insulin sensitivity by suppressing hepatic lipid accumulation and intestinal cholesterol absorption, which subsequently suppressed diet-induced

obesity in mice. For the above reasons, compared to refined grains, whole grain products are beneficial for controlling postprandial glycaemia in people with intolerance to glucose, especially patients with type II diabetes.

Inflammatory damage is another pathway for the progress of type II diabetes. Phytochemicals and dietary fibers have anti-inflammatory effects which may also help reduce the risk and mortality of the type II diabetes. Huang et al. (2018) showed that oral administration of 600 mg /kg body weight /day feruloylated oligosaccharides (FOs) significantly lowered the levels of fasting plasma glucose (FPG), fasting insulin, aspartate transaminase, creatine kinase, and lactate dehydrogenase in rat plasma, and therefore help with the type II diabetes management. FOs are the main form of ferulic acid bound oligosaccharides by esterification that whole use in food have been approved by the U.S. Food and Drug Administration (FDA) (Ishii, 1997). Biskup et al. (2017) found that phytochemicals, such as phytic acid, phenolic acids, and ARs, also contribute to the control of blood glucose levels, insulin sensitivity and hyperinsulinemia in addition to dietary fibers. Qi et al. (2006) found that, after adjustment for age, BMI, lifestyle, and dietary covariates, intakes of both whole grains were associated with significantly decreasing trends of C-reactive protein (CRP) (P for trend = 0.03) and tumor necrosis factor-α receptor 2 (TNF-R_2) (P for trend = 0.017). *In vitro* anti-inflammatory effect of whole wheat extracts has also been reported in a number of previous studies (Bautista-Expósito et al., 2020; Mateo Anson et al., 2011; Tomé-Sánchez et al., 2020; Whent et al., 2012). In summary, consumption of whole grain foods may reduce the risk of inflammation damage and type II diabetes.

1.3.3. Cardiovascular diseases

Several studies showed that subjects who consume three or more portions of foods per day based on whole grain cereals have a 20–30 % lower risk of CVD than subjects with low quantities of whole grain consumption (Gil et al., 2011). The bran fraction other than germ of the whole grains is the major contributor to the reduction of CVD risk. In an animal study, rats were feed with either whole grain bread or refined grain bread, respectively, for 21 days. The results showed that the plasma cholesterol level of the whole wheat group was significantly decreased and the total steroid excretion was significantly enhanced compared to the control group with refined grains (Adam et al., 2003). Yamagata & Yamori (2020) suggested that flavonoids compounds from fruit, vegetables, and whole grains help reduce the risk of CVDs by endothelial dysfunction-related atherosclerosis. Sahu et al. (2019) reported that polyphenolics extract of whole grain wheat attenuated doxorubicin induced cardiotoxicity, which suggested the contribution of phytochemicals to the reduced risk of CVDs. The study further indicated that the polyphenolic extract exhibited better cardio-protective capacity over pure *trans*-ferulic acid and apigenin. Taken together, dietary fibers and phytochemicals in whole grain may contribute to the association between whole grain consumption and reduced risk of CVDs.

1.3.4. Colorectal cancer

The association between increased intake of whole grains and reduced risk of colorectal cancer has been confirmed by epidemiological studies. A prospective study starting at 1999 including 50,118 men and 62,031 women found that men in the highest vs. lowest quintile of whole grain consumption had a 43% lower risk of colorectal cancer (HR = 0.57, 95% CI 0.35–0.93, P trend = 0.04) though no significant trend was found between women (Um et al., 2020). A meta-

analysis by (Benisi-Kohansal et al., 2016) suggested an inverse association between whole-grain intake and colorectal cancers. Dietary fiber and phytochemicals may contribute to this effect. Fiber content may contribute to the increase of stool mass and production of short-chain fatty acids (SCFAs). Studies found that patients with colorectal cancer had lower abundances of *Lactobacillus spp*, and other SCFA-producing bacteria, such as the genera *Clostridium* and *Roseburia*. The prebiotic effect of wheat phenolics have also been reported (Costabile et al., 2008; L. Gong, Chi, Zhang, et al., 2019). Kyrø et al. (2014) reported that high concentrations of plasma ARs were associated with a lower incidence of distal colon cancer. Another unique contribution of whole grain consumption to the colon health may be attributed to its insoluble-bound ferulic acid and di-ferulic acid (Liu, 2007). These phenolic acids exist in insoluble-bound forms and therefore can survive upper gastrointestinal digestion and reach the colon. The gut microbiota may release these phenolic acids so they can exhibit their antioxidative, anti-inflammatory, and anti-proliferative potential in colon. In general, fiber content and unique distribution of phytochemicals of whole grains may explain the relation between whole grain consumption and reduced risk of colon cancer. **Table 1-3** summarizes recent publications on health benefits of whole grain phytochemicals and the potential mechanisms.

1.4. Effect of genotypes and growing environments

Phytochemical profile and antioxidant activity of wheat grains are affected by both wheat genotypes and growing environments. There are many environmental factors such as temperature, precipitation level, fertilizers, and nutrient level in the soil. Genotype (G) x environment (E) interaction also influence wheat phytochemicals. Li et al. (2008) reported

phenolic acid composition of 175 wheat lines grown at the same location in the year of 2005. Their results showed that the highest concentration of total phenolic acids was 1171 μg/g with average levels of 658 μg/g. Winter wheats displayed a range of >3.5-fold but spelt genotypes displayed the narrowest (1.9-fold) range of total phenolic acid concentration. Gotti et al. (2018) analyzed phenolic acid concentration of 5 modern wheat lines and five ancient wheat lines. The results showed that ancient genotypes contain significantly higher concentrations of phenolic acids. Similarly, Loreto et al. (2018) analyzed phenolics of 13 ancient genotypes and 9 modern genotypes. Their results also showed that ancient genotypes contained higher concentration of phenolic acids and demonstrated higher antioxidant activities. In summary, these results demonstrated that there is considerable diversity by genetics in phenolic acid composition and that it may be possible to selectively breed genotypes with higher concentration of phenolic acids.

Fernandez-Orozco et al. (2010) grown 26 wheat genotypes in Hungary for three consecutive years and at three additional countries for the last year. Analysis of phenolic acids suggested that growing location and year significantly influenced phenolic acids concentration, especially for the soluble forms of phenolic acids. But some wheat genotypes were more resistant to changes of environments which suggested the existence of G x E interaction. Bellato et al., (2013) found that genotypes and environments affected concentration of ARs, TPC and antioxidant activities of durum wheats grown in Italy. Ma et al. (2015) found that irrigation level, nitrogen fertilizer application and their interactions had a significant effect on TPC, antioxidant activities and phenolic acid composition of wheat grains. Lu et al. (2015) analyzed phytochemical profiles of 10 wheat genotypes grown at 4 different locations. The results indicated significant effect of G,

E, and G x E. Environment factors had stronger effect than genotype and G x E interaction on TPC, ABTS and DPPH antioxidant activities and soluble phenolic acids. Martini et al. (2014) studied phenolic acids and antioxidant activities of 3 durum wheat varieties grown at several locations for 2 consecutive years. Their results indicated that antioxidant activities and soluble-free phenolic acid were mostly influenced by year, whereas soluble-conjugated and insoluble-bound phenolic acids were mostly influenced by environment × year and genotype, respectively. It seems that soluble phenolic acids were more likely to be affected by environmental factors while insoluble phenolics were more likely to be affected by wheat genotypes. Drawback of current studies is the abusage and lack of detail of the term "environment". Wheat lines growing at different years and locations are subjected to different temperature, precipitation, sunlight and even different levels of pest attack. Future studies are advised to specify the environmental conditions. This will advance the research on factors affecting wheat phytochemical profile and antioxidant activities. Overall, wheat genotypes, growing environment, and their interaction can significantly influence phytochemical profiles of wheat grains (**Table 1-4**).

1.5. Effect of processing

1.5.1. Milling

Distribution of phytochemicals and antioxidant activities in different milled fractions has been thoroughly described in previous studies. Adom et al. (2005) evaluated phytochemicals and hydrophilic and lipophilic antioxidant activities in milled fractions (endosperm and bran/germ) of three wheat genotypes. Their results suggest that in whole-wheat flour, the bran/germ fractions contributed 83% of the TPC, 79% of the total flavonoid content (TFC), 51% of the total lutein, 78% of the total zeaxanthin, 42% of the total β-cryptoxanthin, 85% of the total

hydrophilic antioxidant activity, and 94% of the total lipophilic antioxidant activity. Liyana-Pathirana & Shahidi (2007) examined antioxidant activities of milling fractions from bread wheat and durum wheat. Their results also indicated that bran fractions possessed the highest antioxidant activity whereas the endosperm fraction showed the lowest antioxidant activity in both bread wheat and durum wheat. Using a gradual milling system, Hung & Morita (2008) prepared 16 milling fractions of whole buckwheat grains and analyzed their antioxidant activities. The results showed that the flour milled from the outer layers of buckwheat grains contained higher concentration of phenolic compounds and also stronger antioxidant activities. Spaggiari et al. (2020) confirmed that concentration of phenolic compounds in bran is higher than other milling fractions. Furthermore, they found that milling and refining process resulted in a significant reduction of the amount of phenolic acids and methyl-donors in the end-products. In summary, it has been well established that bran fractions contained much higher concentrations of major phytochemicals and stronger antioxidant activities than flour fractions. This conclusion supported the potential health benefits of whole grain foods.

Different milling process and protocols influence the particle size of whole grain flour or the bran fraction, which future affect the extractability and bioaccessibility of phenolics and their antioxidant properties. Beta et al. (2005) compared TPC and antioxidant of pearled wheat and roller-milled fractions. The results indicated that pearling was an effective technique to obtain wheat bran fractions enriched with phenolic antioxidants. Brewer et al. (2014) evaluated the effect of bran particle size on extractability of bran phytochemicals and antioxidant properties. Their results suggested that wheat bran particle size affected the extractability of phytochemicals and the antioxidant activities as phenolic acids, anthocyanins, carotenoids and ORAC value

increased as the average particle size decreased. It seems that smaller particle sizes are positively related with higher extraction rate. However, intensive milling procedure that led to smaller particle sizes will also lead to decreased total amount of phytochemicals. Future studies are needed to developed processing technique that can make flours with smaller particle sizes and un-damaged phytochemical compositions. Also, higher extractability rate for solvent extraction does not necessarily mean higher bioaccessibility in the human digestive tract. Future studies are needed to evaluate effect of milling/particle sizes on bioaccessibility of wheat phytochemicals in the digestive tract.

1.5.2. Germination

It has been found that germination could affect nutraceutical values of whole grains. (Yang et al, 2001) found that concentration of ferulic acid and vanillic acid decreased slightly on day one of germination then gradually but significantly increased to maximum levels on day seven of germination after 24 or 48 hours of steeping. Similarly, Žilić et al. (2014) found that TPC, free radical scavenging activity, and concentration of *trans*-ferulic acid, *para*-coumaric, and caffeic acid increased after five days of germination. Hung et al. (2011) reported changes of phenolic acids concentration as well as DPPH radical scavenging activity after 24 hours of steeping and two days of germination. They also found that TPC decreased after two days of germination for all the varieties whereas insoluble-bound ferulic acid decreased in one variety and increased in other varieties. Kim et al. (2018) reported that phenolic acids, γ-aminobutyric acid (GABA), and antioxidant activities increased with increasing germination time from 0 hour to 96 hours. The GABA concentration was the highest (39.98 mg/100 g grain) after 96 h of germination. The ORAC values was 1.97 times higher after 96-hour germination than that for the control. Most

studies agreed that long-time germination increased concentration of phytochemicals. However, another study found that short-time germination (less than 24 hours) decreased TPC, radical activities, metal-chelating activities and phenolic acid concentrations of wheat grains (Tian et al., 2019). In summary, long-time germination enhanced nutraceutical values of wheat grains but at the expense of end-use properties. A mixture of germinated flour and un-germinated at a proper ratio may exhibit good end-use properties and enhanced nutraceutical values.

1.5.3. Fermentation

Fermentation processes play a key role in the production of whole grain foods due to its impact on the sensory properties (e.g., taste, aroma, and texture) and potential bio-accessibility of wheat phytochemicals. The term "fermentation" can refer to the general fermentation process by different microorganism strains or specifically to the yeast/sourdough fermentation as a step in bread-making process. Zhai et al. (2015) reported that solid state fermentation with basidiomycete *Agaricus blazei* increased total phenolic content and DPPH radical scavenging activity and superoxide anion radical scavenging ability of whole grains. Similarly, Đorđević et al. (2010) investigated effects of fermentation by two types of microorganisms (lactic acid bacteria *Lactobacillus rhamnosus*, and yeast *Saccharomyces cerevisiae*) on phenolic content and antioxidant activities of cereal grains. Their results also suggested that microorganism activities enhanced TPC, DPPH activity, and ferric ion-reducing antioxidant power (FRAP) of cereal grains. Another study on solid state fermentation of four fungi (*spergillus oryzae* NCIM 1212, *Aspergillus awamori* MTCC No. 548, *Rhizopus oligosporus* NCIM 1215 and *Rhizopus oryzae* RCK2012) found that a 14-fold improvement in soluble TPC (11.61 mg GAE/g grain) was observed in *A. oryzae* fermented wheat, while soluble extracts of *R. oryzae* fermented wheat

exhibited a maximum of 6.6-fold and 5.0-fold enhancement of DPPH radical scavenging activity (8·54 μmol TE/g grain) and ABTS radical scavenging activity (19·5 μmol TE/g grain). Though most studies reported increases in TPC and antioxidant activities, this is not always the case, as decreases in these values have also been reported. Ripari et al. (2019) conducted a fermentation study with 114 bacteria strains and reported that some phenolic acids such as *trans*-ferulic acid were decreased during the fermentation process possibly due to metabolism of phenolic acids by bacteria strains and the activities of decarboxylases, esterases, and reductases enzymes. Generally, it should be emphasized that wheat phenolics exist in both soluble and insoluble fractions. Some studies on fermentation only consider fractions. Bacteria and yeast fermentation can release some phenolic compound from its insoluble-bound fraction. These released phenolics are subject to metabolism activities of microorganisms. And some wheat varieties can be more resistant to the fermentation process. The fermentation process may also change the extractability of insoluble phenolics. For studies evaluating fermentation effects, both soluble fractions and insoluble fractions should be characterized. Biotransformation for phenolic compounds in fermented wheat foods are dependent on microbial strains, cereal grain species, and flour source (Ferri et al., 2016).

Changes of phenolic profile and antioxidant activities of wheat grains during the bread-making fermentation has also been described. Tian et al. (2021b) investigated the effect of yeast fermentation on phenolic acid composition and antioxidant of four winter wheat varieties. The results showed that mixing and fermentation process increased concentration of soluble phenolic acids, TPC, and ABTS/DPPH/ORAC antioxidant activities, while the fermentation process did not significantly influence the insoluble-bound phenolics. Antognoni et al. (2019) reported

changes in carotenoids, phenolic acids and antioxidant capacity in wheat bread doughs fermented with different lactic acid bacteria (LAB) strains and found that some LAB strains caused in situ changes, significantly increasing the concentrations of bio-active compounds in doughs during fermentation. This could improve the functional properties of bakery products by a high concentration in phenolic acids, carotenoids and other bioactive compounds. Koistinen et al. (2016) investigated fermentation effects with both baker's yeast and common sourdough starters including *Candida milleri*, *Lactobacillus brevis* and *Lactobacillus plantarum* using non-targeted metabolic profiling with LC–Q-TOF–MS instrument. The study identified 118 compounds with significantly increased concentrations in sourdoughs and 69 compounds with significantly decreased concentrations in sourdoughs. Compounds with increased concentrations included branched-chain amino acids, metabolites of phenolic acids by microorganisms and other potentially bioactive compound; compounds with decreased concentration included phenolic acids precursors, nucleosides and nucleobases. Moore et al. (2009) investigated effect of pizza crust fermentation on phenolic profiles of two winter wheat varieties. Pizza crust fermentation was performed at low temperature for an extended time (48 hours). Interestingly, their results showed that fermentation did not significantly affect soluble-conjugated ferulic acid concentration and ABTS/ORAC antioxidant activities. It was possible that low temperature inhibited microorganism activities, so they did not release bio-active compounds from the insoluble fractions. Yu & Beta (2015) investigated effect of fermentation on purple wheat and results showed that soluble TPC significantly ($p < 0.05$) increased during mixing and fermentation, from 105.4 to 113.2 mg ferulic acid equivalence (FAE)/100 g. Insoluble-bound phenolics slightly decreased after 30 min fermentation but increased significantly ($p < 0.05$) after 65 min of fermentation. In summary, previous studies generally agreed that fermentation process

increased concentration of soluble phenolic acids and antioxidant activities but effects of fermentation on insoluble phenolics were inconsistent among different studies. Fermentation condition (temperature, time, and strains) as well as wheat genotypes may also influence the effects of fermentation.

1.5.4. Enzymatic treatments

Previous studies have reported that enzymatic treatments can positively influence nutraceutical values of wheat grains. Moore et al. (2006) examined solid-state treatments with Viscozyme L, Pectinex 3XL, Ultraflo L, Flavourzyme 500L, Celluclast 1.5L, and porcine liver esterase on release of phenolic antioxidants from wheat bran. The results indicated that enzymatic treatments can release phenolic antioxidants such as *trans*-ferulic acid from insoluble-bound fractions and consequently increase ABTS/DPPH/ORAC antioxidant activities. Ultraflo L was found to be the most effective and released over 50% of the insoluble *trans*-ferulic acid into the soluble fraction. Likewise, Xue et al. (2020) reported that β-endoxylanase and α-arabinofuranosidase treatments increased soluble phenolic acids concentration and antioxidant activities of wheat brans. This study further showed that brans prepared by enzymatic treatments had enhanced technological properties. Wheat phenolic antioxidant mainly exist in insoluble forms bounded with cell wall materials. Enzymatic treatments can partially break down the network and therefore release phenolic acids into soluble fractions. Currently, concentration of insoluble-bound *trans*-ferulic acid is widely determined by NaOH hydrolysis. However, NaOH hydrolysis may not be able to release all phenolic acids even at an extended hydrolysis time. For studies that are interested in concentration of insoluble-bound phenolic acids, new protocols combing enzymatic treatments

and conventional NaOH hydrolysis may advance the understanding of phenolic acids-cell wall materials interactions.

1.5.5. Baking and other thermal treatments

Thermal processing is a practical method that increased the nutraceutical values of both whole wheat grains and wheat brans. Călinoiu & Vodnar, (2020) reported that thermal processing of wheat brans at 80 °C for 10 min coupled with ultrasound-assisted extraction enhanced TPC, *trans*-ferulic acid, vanillic acid, *para*-coumaric acid of wheat brans by 22.49%, 39.18%, 95.68%, 71.91%, respectively. Randhir et al. (2008) also reported that thermal processing via autoclaving increased TPC and antioxidant activities of wheat, buckwheat and corn. On the contrary, Li et al. (2007) reported that thermal processing did not significantly change TPC, ABTS and ORAC antioxidant activities of purple wheat brans. This might be because that anthocyanins in purple wheat were damaged during the processing though other phenolics were released. Therefore, the net effect was that no significant changes were observed after thermal processing.

Baking process is an important step in bread-making procedures and should be considered as a special case of thermal processing. It is of vital significance to understand effect of baking (and other food-making steps) on phenolic profile and antioxidant activities of wheat grains. Lu et al. (2014) reported that baking process did not significantly influence the concentration of total *trans*-ferulic acid for both refined flour and whole grain flour. Differently, Yu & Beta (2015) reported that bread-making process significantly increased soluble phenolic acids and antioxidant activities but slightly decreased insoluble-bound phenolic acids and antioxidant activities. Tian et al. (2021a) investigated baking effects on four different winter wheat varieties and found that

bread-making process significantly increased soluble phenolic acids and antioxidant activities but also slightly increased insoluble-bound phenolic acids and antioxidant activities. Increase of the soluble TPC and antioxidant activity was partially from Maillard reaction products (MRPs) because bread curst exhibited higher TPC and antioxidant activities than bread crumb. This observation was in agreement with a previous study (Gélinas & McKinnon, 2006). Incorporation of phenolic acids into MRPs were also observed and confirmed by LC-QTOF-MS/MS analysis. Typical findings published in recent years are summarized in **Table 1-5**.

1.6. Gastrointestinal digestion and colon fermentation

To fully understand health benefits of phytochemicals, it is important to understand their potential bioaccessibility and bioavailability. Though studies have found effects of wheat variety, environment and processing on phytochemical profiles and antioxidant activities, potential bioaccessibility of wheat antioxidants has not been fully understood. Liu, (2007) stated that most insoluble-bound wheat phenolics were of low bioaccessibility in the upper gastrointestinal tract and therefore can reach the colon. Colon microbiota are believed to release phenolic acids from the food matrix by fermentation activities. As a result, wheat phenolics are especially beneficial to the colon health compared to the phytochemicals from fruits and vegetables. This may partially explain the association between whole grain consumption and reduced risk of colon cancer. The interaction between undigested phytochemicals and gut microbiota is also worth further investigating.

Some *in vitro* models have been developed to simulate the digestive effects on phytochemicals of whole grain food products. These models can be divided into static models and dynamic models.

Dynamic models provide better mimic to human digestive tract. Common dynamic models include TNO Intestinal Model, in vitro Digestion System (IViDiS), Human Gastric Simulator (HGS) (Thuenemann, 2015). However, dynamic models are expensive and therefore not available to most labs. Static models are less expensive and easy to perform (Brodkorb et al., 2019). Podio et al. (2019) investigated effects of *in vitro* digestion on phenolic profile and antioxidant activity of whole wheat pasta and found that gastric digestion significantly increased soluble TPC and concentration of phenolic acids. But a following intestinal digestion did not further release phenolic compounds from the insoluble-bound fraction. Gong et al. (2019) evaluated effect of simulated digestion and fermentation on potential bio-accessibility of nine different phenolic acids. Their results showed that most phenolic acids in the wheat food matrix had a higher bio-accessibility after the in vitro digestion step (average 57.7–82.1%), than after in vitro colonic fermentation (21.9– 47.5%). Gong et al. (2019) investigated effect of *in vitro* digestion on phytochemical profiles and cellular antioxidant activity of whole rains including wheat, corn, oat and rice. Their results indicated that TPCs of whole grains mostly existed in the digested fractions. The proportion of digested phenolics in total phenolics ranged from 57.7% in corn to 79.6% in oat. However, most phenolic acids were still in the insoluble-bound forms. Therefore, the increase in TPC and cellular antioxidant activities may not only come from phenolics and other phytochemicals. It is possible that the simulated digestion proves also release bio-active peptides that also exhibit antioxidant activities as described by previous studies on antioxidant activities of peptides (Hu et al., 2020; Xu et al., 2019). In general, there are still less amount of literature reports on effect of upper gastrointestinal digestion on potential bio-accessibility of wheat phytochemicals. Hydrolyzed bio-active peptide and sugars may interfere with the results from colorimetric assays. However, these bioactive peptides may also contribute

to the potential health benefits of whole grains and therefore should not be neglected. More future studies are needed to systematically investigate these changes and understand the contribution from both phytochemicals and bioactive compounds generated through hydrolysis of proteins and starches.

Effect of colon fermentation on bio-accessibility of whole grain phytochemicals has also been described in several previous studies. Hole et al. (2012) examined effect of probiotic fermentation on whole grain phenolic acids. Their results showed that fermentation with three probiotic strains, *Lactobacillus johnsonii* LA1, *Lactobacillus reuteri* SD2112, and *Lactobacillus acidophilus* LA-5 increased bioaccessibility of phenolic acids from 2.55 to 69.91 µg/g grain and 4.13 to 109.42 µg/g grain in whole grain barley and oat groat, respectively. Andreasen et al. (2001) reported that human colonic microflora contained esterase activity that can release di-ferulic acids from dietary cereal brans. Furthermore, Braune et al. (2009) found that 8-O-4-DFA can be completely degraded by intestinal microbiota. Ferulic acid and 3-(3-hydroxy-4-methoxyphenyl) pyruvic acid were formed in the transition and finally converted to homovanillic acid, 3-(3,4-dihydroxyphenyl) propionic acid, and 3,4-dihydroxyphenylacetic acid.
Prebiotic effect of whole grains has also been confirmed by previous studies. Gong, et al. (2019) compared prebiotic effect of whole grains and refined grains. The study suggested that whole grains played modulatory effects on gut microbiota by stimulating the growth of beneficial bacteria (*Acidaminococcus*, *Bifidobacterium*, *Lactobacillus*) and inhibiting growth of pathogenic bacteria (*Escherichia*, *Clostridium*, *Streptococcus*). Another study also revealed the prebiotic effect of whole grain products (Costabile et al., 2008). The relationship between consumption of whole grain and colon health worth more in-depth studies.

1.7. Conclusions and future perspectives

In the review chapter, phytochemical composition of whole wheat grains and their potential health benefits were summarized. Wheat genotypes, environmental factors, wheat management and postharvest processing procedures affect the phytochemical profile and antioxidant activity of whole grain flours. Wheat phytochemicals exist in both soluble and insoluble forms, future studies are warranted to analyze both soluble and insoluble fractions while some current studies only evaluated the soluble fractions. Furthermore, bio-accessibility and bioavailability of whole grains should be thoroughly investigated. Absorption, distribution, plasma activities and molecular metabolisms of wheat phytochemicals should be studied. Developing screening methods for fast determination of wheat phytochemicals will also benefit the breeding programs targeting at wheat genotypes with enhanced nutraceutical values. These investigations are important for the production and consumption of whole grain products for improved human health.

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Figures and tables

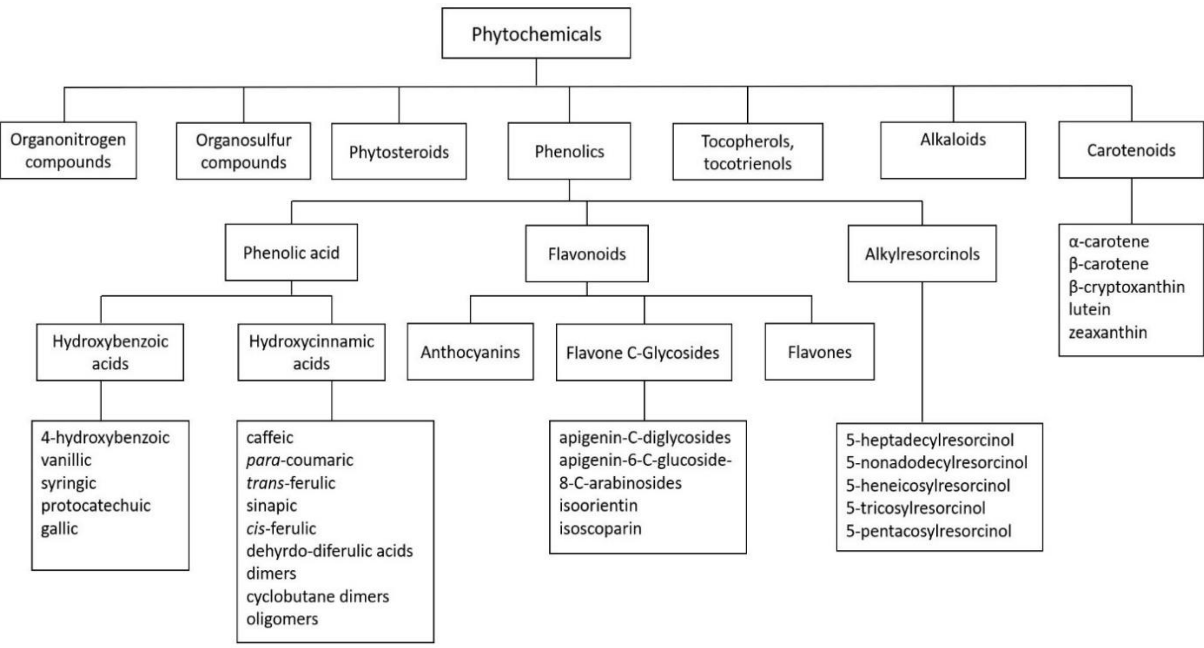

Figure 1-1 Common phytochemicals in cereal grains.

Table 1-1 Common monomeric phenolic acids in whole wheat.

Name	Structure	Soluble (µg/g)	Insoluble-bound (µg/g)
Hydroxycinnamic acids			
trans-ferulic acid		9.4-70	162 to 721
para-coumaric acid		1.7 to 12.1	2.9 to 19.1
sinapic acid		19.1 to 128.0	13.6 to 36.6
Hydroxybenzoic acids			
vanillic acid		7.0 to 24.5	1.7 to 9.0
syringic acid		3.8 to 22.2	1.0 to 13.4
4-hydroxybenzoic acid		2.0 to 11.1	0.2 to 8.6

2,4-hydroxybenzoic acid		7.6 to 116	0 to 215

Table 1-2 Other major phytochemicals in whole wheat.

Phytochemical Group	Example	Analytical Method	Concentration
di-ferulic acids	5-5 DFA	chromatographic separation ; semi-quantitative estimation	7.0 to 51.0 (µg/g)
flavonoids	apigenin	Al^{3+} assays	20.1 to 67.7 mg catechin equivalence/g
natural vitamin E	α -tocopherol	chromatographic separation; quantification by standards	13.6 to 36.6 (µg/g)
carotenoids	lutein	chromatographic separation; quantification by standards	1.3 to 1.8 (µg/g)
alkylresorcinols	5-heptadecylresorcinol	chromatographic separation; quantification by standards	200 to 1732 (µg/g)

Table 1-3 Health benefits of wheat phytochemicals.

Health benefits	Phytochemicals	Experiments	Possible mechanisms	References
Weight control	Alkylresorcinols	Human	Reduced postprandial glucose response associated with increased plasma alkylresorcinols concentration	(Karl et al., 2017)
Diabetes management	Feruloylated oligosaccharides (main form of ferulic acid bound oligosaccharides by esterification)	Rat	decreased levels of fasting plasma glucose (FPG), fasting insulin, aspartate transaminase, creatine kinase, and lactate dehydrogenase in rat plasma	(Huang et al., 2018)
Reduced risk of cardiovascular diseases	Phenolic acids and flavonoids	Rat	attenuated doxorubicin induced cardiotoxicity	(Sahu et al., 2019)
Reduced risk of cardiovascular diseases	Alkylresorcinols	Mice	increased fecal cholesterol excretion by 39.6%; reduced blood cholesterol concentrations by	(Oishi et al., 2015)

			30.4%, enhanced expression of hepatic cholesterol synthetic genes	
Reduced risk of colorectal cancer	Alkylresorcinols	*In vitro* cell experiments	Synergistic effect between fiber microbial metabolite butyrate	(Zhao et al., 2019)
Reduced risk of colorectal cancer	Phenolic acids	*In vitro* cell experiments	Prebiotic effects	(Gong et al., 2019)

Table 1-4 Effects of genotype and environments on phytochemical profiles of whole grains.

Factors	Samples	Results	References
Genotypes	5 ancient and 5 modern wheats under same condition	Ancient wheats contained higher concentration of phenolic acids	(Gotti et al., 2018)
Genotypes	13 ancient and 9 modern genotypes	Ancient wheats contained higher concentration of phenolic acids	(Loreto et al., 2018)
Genotypes	175 wheat genotypes	Durum wheats contained higher soluble phenolic acids; winter wheats contained higher insoluble-bound phenolic acids; huge genetic diversity (>4 fold) in wheat genotypes	(Li et al., 2008)
Genotypes(G) and environments (E): different locations	10 wheat genotypes in 4 locations	Total carotenoids primarily affected by E (45.7%), significantly correlated with low temperature and precipitation level; total tocopherols primarily affected by G x E (71.6%)	(Lu et al., 2015)

Genotypes(G) and environments(E): different locations and growth year	3 durum wheats grown at several locations for 2 years	Soluble phenolic acids primarily affected by environments; insoluble phenolic acids primarily affected by genotypes	(Martini et al., 2014)
Genotypes(G): einkorn, emmer, spelt and common wheat grain and environments(E): crop year	4 spring einkorn, 4 emmer, 4 spelt and 4 common wheat genotypes cultivated under organic cropping system in two-year trials	TPC was in order einkorn > emmer > common wheat > spelt; higher TPC in 2018, a very dry year	(Zrcková et al., 2019)
Environments (E): Nitrogen fertilizer and irrigation	Four N rates (0, 180, 240, and 300 kg ha^{-1}) combined with irrigation times (I_0, no irrigation; I_1, jointing time irrigation; I_2, jointing+ flowering time irrigation),	An appropriate irrigation and N management improved *in vitro* antioxidant potential and concentration of phenolic acids	(Ma et al., 2015)

Table 1-5 Effect of processing on phytochemical profiles of whole flours/wheat brans.

Treatments	Phytochemicals	Results	Reference
Milling	Phenolic acids, flavonoids, carotenoids	Increase in extracted flavonoids and carotenoids; particle size influences the extraction rate	(Brewer et al., 2014)
Gelatinization	Total ester-linked phenolic acids	Microwave treatment gelatinize starch resulting in decreased extraction yield	(Lu & Luthria, 2016)
Thermal processing	Soluble phenolic acids	ferulic acid +39.18%, vanillic acid +95.68%, apigenin–glucoside +71.96%, *p*-coumaric acid +71.91%	(Călinoiu & Vodnar, 2020)
Extrusion	*In vitro* TPC and DPPH assay	Optimized extrusion condition increased TPC and DPPH values of wheat bran	(Ramos-Enríquez et al., 2018)
Steam flash explosion	Soluble ferulic acids, cellular antioxidant, antiproliferative activity	Increased concentration of soluble ferulic acids; increased antioxidant activity	(Chen et al., 2016)
UV-B radiation	*In vitro* TPC, ABTS and DPPH assays	Total phenolics, DPPH and ABTS values (soluble+ insoluble) significantly increased by 26.3, 25.1 and 12.0%, respectively	(Chen et al., 2019)
Ozone treatment	Soluble TPC ABTS and DPPH	No significant changes	(Alexandre et al., 2018)

Germination (96 hours)	Phenolic acids, γ-aminobutyric acid (GABA)	increased concentration of phenolic acids and GABA	(Kim et al., 2018)
Germination (<24 hours)	Phenolic acids, *in vitro* antioxidant potential	Decreased soluble and insoluble phenolic acids; decreased TPC, ABTS and DPPH values	(Tian et al., 2019)
Solid state fermentation	Soluble TPC, DPPH	Increased TPC and DPPH values	(Zhai et al., 2015)
Solid state yeast fermentation (0 to 6 days)	Soluble phenolic acids and flavonoids	ferulic acid +56.6%, vanillic acid +259.3%, dihydroxybenzoic acids +161.2%, apigenin-glucoside +15.3% (day 3)	(Călinoiu et al., 2019)
Fermentation with 12 different lactic acid bacteria strains	Phenolic acids and carotenoids	2 out of 12 strains increased lutein content; changes of phenolic acids profiles are dependent on different strains	(Antognoni et al., 2019)
Bread yeast fermentation	Soluble and insoluble phenolics	Increased soluble phenolic acids concentration	(Tian et al., 2021)
Sourdough fermentation	Soluble polyphenols and flavonoids	Increased of soluble flavonoids and polyphenols	(Saa et al., 2017)
Sourdough fermentation	Non-targeted profiling	118 compounds with significantly increased concentrations and 69 compounds with significantly decreased concentrations	(Koistinen et al., 2016)

Enzymatic treatments by β-endoxylanase and α-arabinofuranosidase treatments	Phenolic acids	Release of insoluble phenolic acids into soluble fractions	(Xue et al., 2020)
Enzymatic treatments of 13 commercial enzymes	Phenolic acids and *in vitro* antioxidant potential	Ultraflo XL release most phenolic acid from insoluble fraction to soluble fraction	(Bautista-Expósito et al., 2020)
Baking	Phenolic acids; *in vitro* antioxidant potential	Increased soluble phenolics; decreased insoluble phenolics	(Yu & Beta, 2015)
Baking	Phenolic acids	Significantly increased soluble phenolic acids; incorporation of phenolic acids into Maillard reaction products; some increase of insoluble phenolic acids	(Tian et al., 2021)
Kernel puffing	Phenolic acids	Soluble phenolic acids increased; insoluble phenolic acids did not change	(Hidalgo et al., 2016)

Chapter 2 - Phenolic acid composition and antioxidant activity of hard red winter wheat varieties[*]

Abstract

Whole wheat consumption is associated with reduced risk of chronic diseases. This study determined total phenolic content (TPC), total flavonoid content (TFC), free radical scavenging activities, metal chelating activity, and phenolic acid composition of 12 hard red winter wheat varieties. TPC, TFC, antioxidant activity and metal chelating activity varied significantly among different varieties. *Trans*-ferulic acid, syringic acid, vanillic acid, *p*-coumaric acid, sinapic acid, and 4-hydroxybenzoic acid were detected and quantified in free, conjugated and bound fractions. Overall, LCS Mint and WB Grainfield varieties have higher phenolic and flavonoid content. Everest, SY Monument and T158 varieties have stronger antioxidant activity. Positive correlations were obtained between total phenolic acid content and antioxidant activity. Our result suggested that phenolic acid composition and antioxidant activity of hard wheat are highly dependent on their genotypes.

Key words: hard red winter wheat; phenolic acid; radical scavenging activity; metal chelating activity

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2.1. Introduction

Epidemiological studies have supported the role that whole grain products play in reduced risk of chronic diseases (Jonnalagadda et al., 2011b; Masisi et al., 2016; Tang et al., 2015; Tighe et al., 2010) and gastrointestinal pathologies (Gil et al., 2011). The health benefits of whole grain products are in part attributed to their unique antioxidant activities. Antioxidants may prevent damage to important biomolecules such as DNA and proteins through scavenging of free radicals, chelation of transition metals, and activation of antioxidative enzyme activities (Fardet, 2010; Pham-Huy et al., 2008).

Wheat products are staple cereal grain food for most population. Antioxidant activities of whole wheat have been reported by many previous studies (Kim & Kim, 2016a; Liyana-Pathirana & Shahidi, 2007a; Lv et al., 2012; Okarter et al., 2010b; Vaher et al., 2010). Phenolic compounds, especially phenolic acids, are believed to be one of the major contributors to the antioxidant activity of cereal grains (Hung et al., 2011b). Phenolic acids of wheat are uniquely distributed as soluble-free, soluble-conjugated and insoluble-bound forms, while phenolic acids and other bioactive compounds of fruits and vegetables are mainly in soluble-free and soluble-conjugated forms (Adom & Liu, 2002). It is suggested that insoluble-bound fractions of phenolic acids are bound to cell wall materials so they may survive upper gastrointestinal digestion and reach the colon. Therefore, phenolic acids of wheat grains may perform stronger antioxidant activities in the colon than phenolic acids of fruits and vegetables (Liu, 2007).

It’s widely accepted that wheat samples differed in their antioxidant properties. Genotype and growing environment are believed to be the major determinants of the antioxidant of wheat (Abdel-Aal & Rabalski, 2008; Y. Li et al., 2015; Moore, Liu, et al., 2006). Wheat products with optimal antioxidant activity and health benefits can be obtained by growing selected varieties under optimal conditions. Practically, environmental factors such as solar radiation, temperature stress and precipitation level are hard to control and may vary greatly from year to year. Therefore, wheat variety might be the primary consideration for enhanced antioxidant activity of wheat products.

Kansas is one of the leading wheat-production states in the United States. In 2015, wheat production of Kansas accounts for 16 percent of all wheat production and 23 percent of winter wheat production in the U.S. (USDA). To the best of our knowledge, a comprehensive study of phenolic acid composition and antioxidant activity of Kansas-grown wheat varieties is not available. The breeders and food industry may be benefited by a comprehensive understanding of properties of different wheat varieties. Therefore, the objective of this study was to determine the phenolic content, flavonoid content, free radical scavenging activities, metal chelating activities, as well as phenolic acid compositions of 12 Kansas-grown hard red winter varieties.

2.2. Materials and methods

2.2.1. Materials

Twelve hard red winter wheat varieties grown in Healy, Kansas, USA during the 2016 and 2017 crop seasons were collected. All grains were ground to fine flour with a coffee grinder before analysis. Ferulic acid, syringic acid, vanillic acid *p*-coumaric acid, gallic acid, sinapic acid, 4-

hydroxybenzoic acid, Trolox, Folin-Ciocalteu reagent, 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma (St. Louis, MO, USA). Catechin standard and other general chemicals were obtained from Fisher Scientific (Pittsburgh, PA, USA).

2.2.2. Extraction of free phenolic compounds

Free phenolic compounds were extracted according to previous reported protocols with some modifications (Djordjevic et al., 2011). Briefly, 25 g flour was extracted twice with 50mL 80% chilled ethanol for 10 min. The mixture was centrifuged at 3000 xg for 10 min. The supernatants were combined and evaporated to less than 5mL and then adjusted with deionized water to a final volume of 25 mL.

2.2.3. Extraction of bound phenolic compounds

Bound phenolic compounds were extracted according to previously reported protocols with some modifications (Inglett et al., 2011). Briefly, 2 g flour was first extracted twice with 80% chilled ethanol. The residue was digested with 50 mL 2 M NaOH for 3 hours under nitrogen in dark. After centrifugation at 4000 xg for 10 minutes, the supernatant was acidified with 6M HCl to pH 2 and defatted. Then, it was extracted five times with ethyl acetate, and the organic phase was collected and evaporated to dryness under nitrogen. The residue was re-dissolved in 5 mL HPLC-grade methanol.

2.2.4. Determination of total phenolic content

The total phenolic content (TPC) was determined according to a method reported previously (C. Xu et al., 2010). 0.1mL sample extract, 7.9 mL deionized water, and 0.5 mL 2 N Folin-Ciocalteau reagents were mixed. After 5 minutes, 1.5 mL of 20% Na_2CO_3 solution was added. The absorbance was measured at 765nm after 2 hours. The final TPC was converted to µM gallic acid equivalence per gram (µM GAE/g) grain.

2.2.5. Determination of total flavonoids content

The total flavonoid content (TFC) was determined using a colorimetric method reported previously (Chlopicka et al., 2012). 0.1 mL sample extracts were mixed with 2.4 ml deionized water and 0.15 mL 5% $NaNO_2$ solution. After 5 minutes, 0.15 mL $AlCl_3$ solution was added. After 1 minute, 0.5 mL 2M NaOH was added and the mixture was diluted with deionized water to a final volume of 5 mL. The absorbance was measured immediately at 510 nm. The final TFC was converted to µM catechin acid equivalence per gram (µM CE/g) grain.

2.2.6. Determination of DPPH radical scavenging activity

The DPPH assay was determined according to the reported method with some modifications (Pownall et al., 2010). Briefly, 0.5 mL of diluted extract was mixed with 5 mL 0.2 mM DPPH solution. The absorbance was measured at 517 nm after 30 minutes. The DPPH radical scavenging activity was converted to µM vitamin C equivalence per gram (µM VCE/g) grain.

2.2.7. Determination of ABTS radical scavenging activity

The ABTS assay was performed following a previous method (Chanput et al., 2016). 0.1 mL extract was mixed with 3.8 mL diluted ABTS solution. The mixture was kept at dark condition for 10 minutes. The absorbance was measured at 734 nm and quantified using Trolox as the standard. Final result was presented in μM Trolox equivalence per gram (μM TE/g) grain.

2.2.8. Determination of metal chelating activity

Wheat extract with 50% acetone was used for metal chelating activity determination (Yamaguchi et al., 2000). Briefly, 2 g flour was extracted with 25 mL 50% acetone under nitrogen for 24 hours. The mixture was centrifuged at 3500 xg for 10 minutes. The final mixture contained 0.25 mL sample extract, 0.25 mL $FeSO_4$ (1.2mM), 1mL Tris-HCl (1M, pH=7.4), 1mL 2,2′-bipyridyl (0.1% in 0.2M HCl), 0.4 mL hydroxylamine hydrochloride (10%, w/w) and 2.1 ml ethanol. The absorbance was measured at 522nm after 40 minutes and quantified using an EDTA standard curve.

2.2.9. HPLC sample preparation and analysis

To prepare samples for soluble-free fractions, 10 mL free extract was further extracted five times with ethyl acetate. The organic phases were combined and evaporated to dryness under nitrogen. The residue was re-dissolved in 2 mL methanol. To prepare samples for soluble-conjugated phenolic acid analysis, 5 ml free extract was digested with 2 M NaOH for 3 hours under nitrogen at dark condition. The mixture was acidified with 6 M HCl to pH 2 and extracted five times with ethyl acetate. The organic phases were combined and then evaporated to dryness under nitrogen

and re-dissolved in 1ml methanol. Extracts of bound phenolic compounds were directly used for HPLC analysis of insoluble-bound phenolic acid.

Reversed phased-HPLC method was performed with a SHIMADZU (Kyoto, Japan) system equipped with diode array detector (DAD) using a Phenomenex Kinetex F5 column (150 x 4.6mm). The mobile phase A was 0.1% TFA in HPLC grade water. The mobile phase B was HPLC grade acetonitrile. The DAD detector was set to scan signals from 220nm to 800nm. 5μL sample was injected each time and HPLC gradient was programmed as follows: total pump speed was kept constantly at 0.8 mL/minute; 10% mobile phase B was used from sample injection to 10 minutes; percentage of mobile phase B was increased linearly from 10% to 20% from 10 minutes to 17 minutes; and 20% mobile phase B was used from 17 minutes to 22 minutes. The column was equilibrated with 10% mobile phase B for 5 minutes between each injection. Resolved peaks were identified according to their retention times and UV spectrums from DAD. The analysis was performed at room temperature. Quantification of individual phenolic acid was conducted using total area under peak with external standard curves.

2.2.10. Statics analysis

Data from this study were reported as mean ± SD from three replicates for each test. Results were subject to one-way analysis of variance, and differences among means ($p<0.05$) were evaluated using SAS institute’s SAS software, version 9.3 (Cary, NC, USA). Correlation analysis was conducted using two-tailed Pearson correlation test.

2.3. Results and discussions

2.3.1. Phenolic content of wheat varieties

Free and bound phenolic contents of studied wheat varieties are given in **Figure 2-1**. The free phenolic content ranged from 0.88 (LCS Wizard) to 1.55 (Byrd) μM GAE/g. The free phenolic content of Byrd and Tam 204 were significantly higher ($p<0.05$) than other varieties. The bound phenolic content ranged from 5.47 (Jagger) to7.72 (WB Grainfield) μM GAE/g grain. Bound phenolic content of WB Grainfield was significantly higher ($p<0.05$) than other varieties, while bound phenolic content of Jagger was the lowest among all the tested varieties. On average, bound phenolic content accounted for 85% of the total phenolic content. This value was overall in agreement with previous studies (Hung et al., 2011b; Okarter et al., 2010b; Verma et al., 2009).

Andarwulan *et al.* (2010) reported that TPC of 11 vegetable ranged from 1.96 to 8.76 μM GAE/g Fresh Weight. Contreras-Calderón *et al.* (2011) reported that TPC of 33 fruits ranged from 0.92 to 59.88 μM GAE/g Fresh Weight. Comparing with these results, it seemed that TPC of whole wheat was comparable to many vegetables and even some fruits. Therefore, whole wheat could be another important source of natural phenolic compounds.

2.3.2. Flavonoid content of wheat varieties

Flavonoid contents of tested wheat samples were expressed as μM CE/g grain (**Figure 2-2**). Free flavonoid content of tested samples ranged from 0.098 (WB Grainfield) to 0.22 (Jagger) μM/g. Free flavonoid content of WB Grainfield was significantly lower than other varieties ($p<0.05$). Bound flavonoid content of wheat varieties ranged from 1.12 (Jagger) to 2.50 (WB 4458) μM

CE/g. Bound flavonoid content of Jagger, T158, Everest and 2137 were similar and significantly lower ($p<0.05$) than other varieties. Bound flavonoid content of WB Grainfield was significantly higher ($p<0.05$) than other varieties.

Flavonoids are the largest subgroup of phenolic compounds. Fruits, vegetables, legumes and tea are generally considered as major dietary sources for flavonoids (Jun et al., 2016; Kent et al., 2015). Marinova *et al.* (2005) reported total flavonoid content of various fruits and vegetables. The total flavonoid content of fruit ranged from 1.13 μM CE/g (red apple) to 6.58 μM CE/g (blueberry), and the total flavonoid content of vegetables ranged from 0.086 μM CE/ g (spring onion) to 3.35 μM CE/g (lettuce). Comparing with those values, free flavonoids contents of whole wheat were much lower than fruits and most vegetables. However, total flavonoids content (free plus bound) of whole wheat was comparable to many types of vegetables and fruits. This suggests that whole grains could be another important source of flavonoid compounds, and contribution of whole grains in dietary flavonoids intake might have been underestimated.

2.3.3. DPPH radical scavenging activity of wheat varieties

DPPH radical scavenging activities of tested samples were expressed in μM VCE/g grain (**Figure 2-3**). For free fractions of whole wheat, DPPH value ranged from 2.81 (T158) to 4.00 (2137) μM VCE/g. DPPH value of T158 was significantly lower than other varieties ($p<0.05$). DPPH values of 2137, Fuller, and Tam 204 varieties were the highest among all the varieties. For bound fractions of whole wheat, the range of DPPH radical scavenging activities ranged from 23.62 (LCS Mint) to 33.27 (T158) μM VCE/g. DPPH value of T158 and SY Monument varieties were similar and significantly higher ($p<0.05$) than other varieties. DPPH value of

Everest was lower than T158 and SY Monument but significantly higher than other varieties. Our result suggested that there were significant variances of DPPH radical scavenging activities among wheat varieties. Different research groups employed various DPPH assay protocols which differed in DPPH concentrations, incubation times, extraction solvents, pH values and calculation methods. Thus, it's generally not feasible to compare values of DPPH radical scavenging activity from different literatures. In this study, we expressed DPPH radical scavenging capacity as μM VCE/g. Adom and Liu (2002) reported total antioxidant activity of whole wheat varieties as μM VCE/g using a total oxyradical scavenging capacity (TOSC) assay. Interestingly, the range of our reported result was very comparable to the range they reported though different assay protocols were employed.

2.3.4. ABTS radical scavenging activity of wheat varieties

Values of ABTS radical scavenging activity were expressed as μM TE/g grain (**Figure 2-4**). For free fractions of tested samples, the result ranged from 6.42 (LCS Wizard) to 11.82 (Byrd) μM TE/g. ABTS value of Byrd was significantly higher ($p<0.05$) than other varieties evaluated. Everest, T158 and SY Monument had similar free ABTS values that were significantly higher ($p<0.05$) than other varieties except for Byrd. ABTS values of WB Grainfield and LCS Wizard were the lowest. The ABTS results of bound fractions ranged from 42.07 (LCS Mint) to 77.51 (SY Monument) μM TE/g. Except for SY Monument, ABTS values of T158 and Everest were similar and significantly higher ($p<0.05$) than other varieties.

2.3.5. Metal chelating activity of wheat varieties

For the determination of phenolic content, flavonoid content and radical scavenging activity, free and bound fractions were separately extracted and tested. Values of bound fractions were usually much higher than that of free fractions. Our preliminary results found that metal chelating capacity was much higher in free fractions under experimental conditions. Metal chelating activity of wheat samples was expressed as μM EDTA equivalence per gram grain sample (**Figure 2-5**). The value ranged from 1.03 (Tam 204) to 3.15 (Byrd) μM EDTA equivalence/g. Values of Everest and WB Grainfield were similar and significantly lower ($p<0.05$) than other varieties except for Tam 204. Literatures reported much varied range of metal chelation capacity of wheat extracts. Liyana-Pathirana and Shahidi (2007) reported that Fe^{2+} chelation activity of Canadian Western Amber Durum (CWAD) and Canadian Western Red Spring (CWRS) were 2.94±0.12 μM EDTA equivalence/g and 3.06±0.06 μM EDTA equivalence/g, respectively. Moore *et al.* (2005) reported that chelating activities of Maryland soft wheat varieties ranged from 0.38 μM EDTA equivalence/g to 1.35 μM EDTA equivalence/g.

The 2,2′-bipyridyl competition assay has been employed by many studies for determination of metal chelating activity (Liyana-Pathirana & Shahidi, 2007a; Moore et al., 2005b; Yamaguchi et al., 2000). Our preliminary data suggested that mixing order of reagents and time allowed for color development significantly influenced the experimental results (Supporting Information Figure S5). Therefore, consistent timing and mixing order of each reagent was critical for the reproductivity of the measurements.

2.3.6. Phenolic acid composition of wheat varieties

Phenolic acids are derivatives of hydroxybenzoic acid or hydroxycinnamic acid. In our study, ferulic acid, *p*-coumaric acid, vanillic acid, 4-hydroxybenzoic acid and syringic acid were detected in soluble-free, soluble-conjugated and insoluble-bound forms in all the studied wheat varieties. Sinapic acid was detected to be the predominant phenolic acid in soluble-conjugated form. Caffeic acid was not detected under experimental conditions. Ferulic acid, *p*-coumaric acid and sinapic acid are derivatives of hydroxycinnamic acid. Vanillic acid, vanillic acid and 4-hydroxybenzoic acid are derivatives of hydroxybenzoic acid. Phenolic acid contents were expressed as μM phenolic acid/100 g grain. (**Table 2-1**)

Ferulic acid is the predominant phenolic acid found in whole wheat. Adom *et al.* (2003) reported that the total ferulic acid content of 11 wheat varieties ranged from 147.7 to 303.0 μM/100g whole grain. Moore *et al.* (2005) reported that total ferulic acid content of eight soft spring wheat varieties ranged from 234.8 to 320.0 μM/100g sample. Mpofu *et al.* (2006) reported that total ferulic acid content of six Canadian west hard spring wheat ranged from 191.0 to 227.1 μM/100g. Okarter *et al.* (2010) reported that the total ferulic acid content of six diverse varieties of whole wheat ranged from 310.8 to 496.1 μM/100g. Lv *et al.* (2012) reported that total ferulic acid content of 10 Maryland-grown soft winter wheat flours ranged from 96.4 to 165.5 μM/100g. In this study, total ferulic acid of selected hard red winter varieties ranged from 138.07 (SY Monument) to 204.95 (Fuller) μM/100g. Bound ferulic acid content accounted for >95% of the total ferulic acid content. These findings were comparable to those cited above. Some significant differences of total ferulic acid content were detected among selected varieties. Total ferulic acid of Byrd and SY Monument were significantly lower ($p<0.05$) than other tested varieties. The

amount of ferulic acid in soluble-conjugated form ranged from 1.001 (WB Grainfield) to 2.224 (Everest) µM/100g.

The *p*-coumaric acid was found to be the second most predominant phenolic acid in whole wheat. Total *p*-coumaric acid content ranged from 10.56 (WB 4458) to 28.37 (Everest) µM/100g. Moore *et al.* (2005) reported that the *p*-coumaric acid content of eight Maryland-grown soft wheat varieties ranged from 6.34 to 8.60 µM/100g. Mpofu *et al.* (2006) reported that the *p*-coumaric acid content of six wheat varieties ranged from 14.59 to 22.70 µM/100g. Okarter *et al.* (2010) reported that the *p*-coumaric acid content of six diverse wheat varieties ranged from 33.5 to 52.3 µM/100g. Our value was consistent with some studies (Moore et al., 2005b; Mpofu et al., 2006). But our value was lower than the range reported by Okarter *et al.* (2010), in which the bound fraction on average accounted for only 47.3% of the total *p*-coumaric acid. While our study and results from Moore *et al.* (2005) suggested that bound fraction of *p*-coumaric acid contributed to >85% of the total *p*-coumaric content. Based on the above discussion, total *p*-coumaric content and its distribution among different fractions appear to be more diverse than ferulic acid.

Interestingly, sinapic acid was only detected in soluble-conjugated form and it was found to be the most abundant phenolic acid in soluble-conjugated form. Content of sinapic acids ranged from 2.010 (WB Grainfield) to 11.25 (Fuller) µM/100g. Content and distribution of sinapic acid were not reported in many recent studies (Loreto et al., 2018b; Lv et al., 2012; Okarter et al., 2010b; Zhang et al., 2012). Different sample preparations and HPLC protocols might be the reason for the observation.

Derivatives of hydroxybenzoic acid including vanillic acid, syringic acid and 4-hydroxybenzoic acid were also detected in our experiment. Content and distribution of vanillic acid and syringic acid were reported in some recent studies (Li et al., 2008b; Mattila et al., 2005; Moore et al., 2005b; Mpofu et al., 2006; Okarter et al., 2010b). In our study, total content of vanillic acid and syringic acid ranged from 3.31 to 4.17 μM/100g and 3.83 to 5.62 μM/100g, respectively. The total amount of vanillic acid and syringic acid was consistent with previous studies mentioned above. In our study, bound fraction of vanillic and syringic acid contributed most to the total content while most other studies suggested that soluble-conjugated fraction contributed most to the total content. In addition, vanillic and syringic acid were detected in all three fractions among all chosen wheat varieties. This was not consistent with some previous studies (Moore et al., 2005b; Okarter et al., 2010b; Zuchowski et al., 2011). Differences of wheat varieties, growing environment, as well as HPLC sample preparation and HPLC gradient program protocols might be the reason for some inconsistence.

4-hydroxybenzoic acid was detected in soluble-free, soluble-conjugated and insoluble-bound fractions among all the chosen varieties. Total 4-hydroxybenzoic acid content ranged from 2.40 (Tam 204) to 4.03 (Fuller) μM/100g. Bound fraction contributed the most to the total content. Zhou *et al.* (2004) reported that total 4-hydroxybenzoic acid content of Swiss red wheat flour was 3.62 μM/100g. Mattila *et al.* (2005) reported that total 4-hydroxybenzoic acid content of whole wheat flour was 9.42 μM/100g but no detail about contribution of each fraction was provided. Li *et al.* (2008) reported that average 4-hydroxybenzoic content of winter wheat varieties and spring wheat varieties were 5.51 and 6.59 μM/100g, respectively. The total amount

was consistent with our study. However, in the study of Li *et al.* (2008), soluble-conjugated fraction contributed most to the total content which was different from our study. Interestingly, 4-hydroxybenzoic acid was not reported in many recent studies (Okarter et al., 2010b; Revanappa & Salimath, 2011; Wang et al., 2013; Zhang et al., 2012).

Though it was generally believed that phenolic acid was an important source of natural antioxidant of wheat, correlation of phenolic acid composition and antioxidant activity in wheat flour was barely reported. One reason was that besides phenolic acids, there were other natural antioxidants in the testing samples such as flavonoids, carotenoids and tocopherols (Dinelli et al., 2011; Leoncini et al., 2012b). Another reason was that each phenolic acid has different antioxidant potential and there might be unknown interactions between phenolic acids (Vaher et al., 2010). Therefore, total amount of the phenolic acid was used for the correlation study with antioxidant activity. Total free phenolic acid (TSPA) was defined as the sum of the amount of each phenolic acid in soluble-free and soluble-conjugate fractions. Total bound phenolic acid (TBPA) was defined as the sum of the amount of each phenolic acid in insoluble-bound fraction. For correlation analysis, 24 samples (12 free and 12 bound) were analyzed together. Results showed that total phenolic acid was positively correlated with ABTS radical scavenging activity (R^2= 0.785, N=24, $p < 0.01$). Similarly, total phenolic acid was positively correlated DPPH radical scavenging activity (R^2= 0.913, N=24, $p < 0.01$). These results confirmed that phenolic acid was one of major sources of antioxidant in wheat extract.

2.4. Conclusions

Our study demonstrated that antioxidant activities as well as phenolic acid compositions were significantly varied among wheat varieties. Overall, LCS Mint and WB Grainfield varieties seem to have higher total phenolic content. Everest, SY Monument and T158 varieties seem to have stronger antioxidant activity. Correlation analysis suggested that phenolic acids were one of major source of natural antioxidant in whole wheat. Identifying wheat varieties with significant levels of phenolic content and strong antioxidant activity has the potential not only to promote the value-added cultivation and use of wheat rich in these factors, but also to provide health benefit to consumers, thereby enhancing wheat profitability, agricultural economy, and public health.

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Conflict of interest

The authors declared no conflict of interest.

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Table 2-1 Phenolic acid compositions of wheat varieties (mean ± SD, n= 3).

Wheat Varieties	Soluble-free (µmol/100g grain)	Soluble-Conjugated (µmol/100g grain)	Insoluble- Bound (µmol/100g grain)	Total (µmol/100g grain)
Trans-ferulic Acid[a]				
LCS Mint	0.861±0.062a	1.048±0.138e	149.675±1.165cd	152.265±1.288de
LCS Wizard	0.878±0.049a	1.004±0.064e	157.439±2.005c	160.121±2.071d
WB 4458	0.749±0.025bc	1.117±0.049e	171.765±5.377b	174.506±5.411bc
WB Grainfield	0.744±0.038bc	1.001±0.0193e	148.406±1.889cd	150.389±2.035def
Byrd	0.920±0.070a	2.039±0.044ab	134.173±5.259e	138.628±5.291f
Everest	0.741±0.039bc	2.224±0.067a	151.595±7.399cd	157.185±8.081de
SY Monument	0.761±0.019bc	1.926±0.164bc	133.782±0.649e	138.068±0.329f
T158	0.851±0.023ab	1.735±0.026cd	140.301±0.489de	144.371±0.329ef
2137	0.743±0.025bc	1.767±0.011cd	177.072±2.186b	181.076±2.132b
Fuller	0.675±0.024c	2.174±0.097a	198.041±8.725a	204.945±8.703a
Jagger	0.718±0.026c	1.706±0.096cd	158.143±6.509c	162.293±6.313cd
Tam 204	0.721±0.023c	1.548±0.024d	155.065±0.529c	158.520±0.486d
Para-coumaric Acid[b]				
LCS Mint	0.312±0.039c	0.457±0.051ef	10.588±1.507e	11.157±1.128e
LCS Wizard	0.497±0.047b	0.385±0.030f	16.589±1.241c	17.472±1.244cd
WB 4458	0.304±0.055c	0.599±0.159def	9.633±0.059e	10.559±0.163e
WB Grainfield	0.304±0.055c	0.465±0.042f	18.301±1.702bc	19.049±1.657bc
Byrd	0.299±0.002c	1.020±0.066bc	16.165±2.370cd	17.095±1.819cd
Everest	3.427±0.047a	2.675±0.088a	22.403±1.654a	28.370±1.212a
SY Monument	0.287±0.016c	0.776±0.173cde	10.905±1.442e	11.969±1.607e
T158	0.350±0.044c	1.133±0.174b	11.215±0.983e	12.699±0.972e
2137	0.362±0.024c	0.487±0.001ef	20.636±0.570ab	21.481±0.586b
Fuller	0.346±0.012c	0.750±0.104def	17.599±1.715bc	18.612±1.609bc
Jagger	0.269±0.006c	0.545±0.057def	12.835±1.514de	13.746±1.647de
Tam 204	0.306±0.028c	0.819±0.037bcd	16.454±0.520cd	17.586±0.516c
Sinapic Acid[c]				
LCS Mint	ND	2.362±0.124de	ND	2.362±0.124de
LCS Wizard	ND	2.434±0.075de	ND	2.434±0.075de
WB 4458	ND	2.633±0.035cde	ND	2.633±0.035cde

WB Grainfield	ND	2.010±0.063de	ND	2.010±0.063de
Byrd	ND	4.960±0.246cd	ND	4.960±0.246cd
Everest	ND	8.171±1.999b	ND	8.171±1.999b
SY Monument	ND	4.435±1.562cde	ND	4.435±1.562cde
T158	ND	4.619±0.089cde	ND	4.619±0.089cde
2137	ND	4.621±0.153cde	ND	4.621±0.153cde
Fuller	ND	11.247±2.310a	ND	11.247±2.310a
Jagger	ND	5.408±0.300bc	ND	5.408±0.300bc
Tam 204	ND	3.684±0.169cde	ND	3.684±0.169cde
Syringic Acid[d]				
LCS Mint	0.329±0.010d	0.536±0.092de	3.970±0.083ab	4.835±0.084bc
LCS Wizard	0.323±0.007de	0.374±0.059e	3.128±0.184bc	3.825±0.0184c
WB 4458	0.357±0.008d	0.837±0.048cd	2.927±0.234bc	4.121±0.234bc
WB Grainfield	0.329±0.009bc	0.547±0.049de	4.080±0.075a	4.955±0.075ab
Byrd	0.438±0.005a	1.539±0.268ab	2.819±0.131bc	4.797±0.131ab
Everest	0.447±0.013a	1.836±0.099a	2.764±0.044c	5.047±0.044ab
SY Monument	0.394±0.002b	1.573±0.065ab	2.840±0.007c	4.807±0.006ab
T158	0.401±0.000b	1.310±0.065b	2.897±0.267c	4.608±0.267bc
2137	0.304±0.004ef	0.786±0.092cd	3.306±0.126bc	4.397±0.125bc
Fuller	0.289±0.003f	1.535±0.164ab	3.800±0.137ab	5.623±0.137a
Jagger	0.300±0.008f	0.911±0.007c	3.051±0.048bc	4.262±0.048bc
Tam 204	0.401±0.007b	0.976±0.011c	2.922±0.150bc	4.299±0.015bc
Vanillic Acid[e]				
LCS Mint	0.595±0.009a	0.813±0.011g	2.388±0.011b	3.797±0.048cd
LCS Wizard	0.552±0.016c	0.695±0.020h	2.160±0.012de	3.407±0.071fg
WB 4458	0.539±0.007bc	0.915±0.018f	2.287±0.017cd	3.741±0.186d
WB Grainfield	0.568±0.004cd	0.742±0.007h	2.729±0.028a	4.038±0.100b
Byrd	0.464±0.000e	1.616±0.039a	2.089±0.072ef	4.169±0.154a
Everest	0.537±0.004h	1.203±0.010c	1.778±0.014j	3.340±0.072bc
SY Monument	0.445±0.004d	1.370±0.022b	1,931±0.009h	3.837±0.143c
T158	0.360±0.002f	1.321±0.007b	2.232±0.016cd	4.000±0.143b
2137	0.353±0.001h	0.997±0.009e	1.850±0.006ij	3.305±0.058h
Fuller	0.386±0.000h	1.143±0.008d	1.972±0.011hg	3.468±0.012f
Jagger	0.397±0.002g	1.051±0.017e	1.922±0.010hi	3.360±0.021g

Tam 204	0.401±0.007g	1.173±0.017cd	2.024±0.014fg	3.395±0.013e
4-Hydroxybezonic Acid[f]				
LCS Mint	0.460±0.036a	0.428±0.027e	1.726±0.015cd	2.614±0.015fg
LCS Wizard	0.388±0.006b	0.407±0.020e	1.788±0.028c	2.583±0.037fg
WB 4458	0.358±0.049b	0.574±0.088e	1.622±0.023e	2.555±0.158fg
WB Grainfield	0.333±0.006bc	0.417±0.031e	2.342±0.058a	3.091±0.053cd
Byrd	0.266±0.001d	1.161±0.043c	1.275±0.049g	2.702±0.087f
Everest	0.203±0.001e	1.812±0.015a	1.606±0.034e	3.620±0.045b
SY Monument	0.358±0.001b	1.186±0.093c	1.449±0.038f	2.993±0.106de
T158	0.270±0.002d	1.025±0.095cd	1.500±0.030f	2.795±0.124ef
2137	0.224±0.020de	1.107±0.044c	1.973±0.020b	3.304±0.023c
Fuller	0.168±0.007e	1.557±0.132b	2.304±0.030a	4.030±0.114a
Jagger	0.176±0.012e	0.894±0.046d	1.682±0.023de	2.752±0.012ef
Tam 204	0.277±0.013cd	0.879±0.059d	1.248±0.021g	2.404±0.069g

Note: Different letters within each column indicate significant differences ($p<0.05$); ND= not detected.

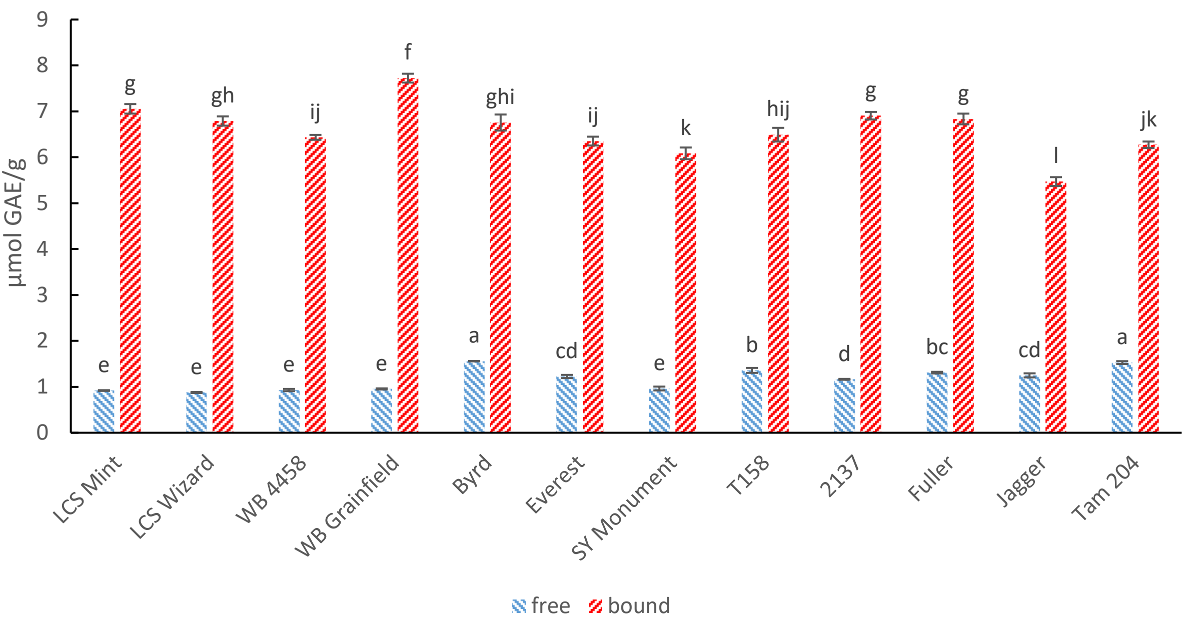

Figure 2-1 Total phenolic content of wheat varieties.

Bars with different letters are significantly different ($p<0.05$).

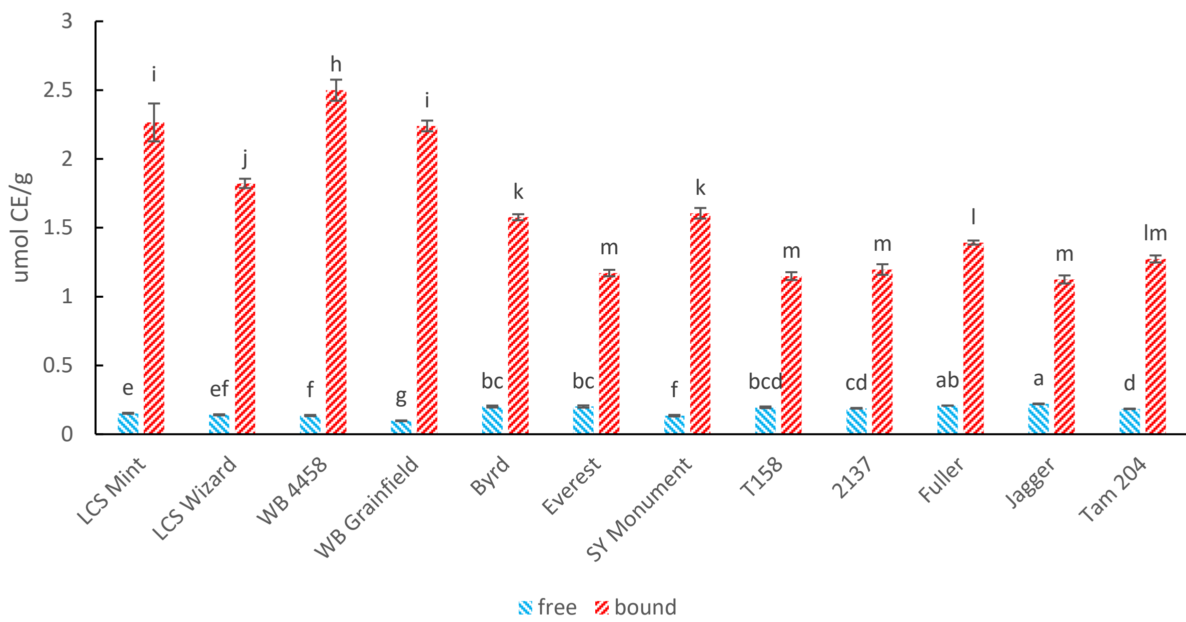

Figure 2-2 Total flavonoids content of wheat varieties.

Bars with different letters are significantly different ($p<0.05$).

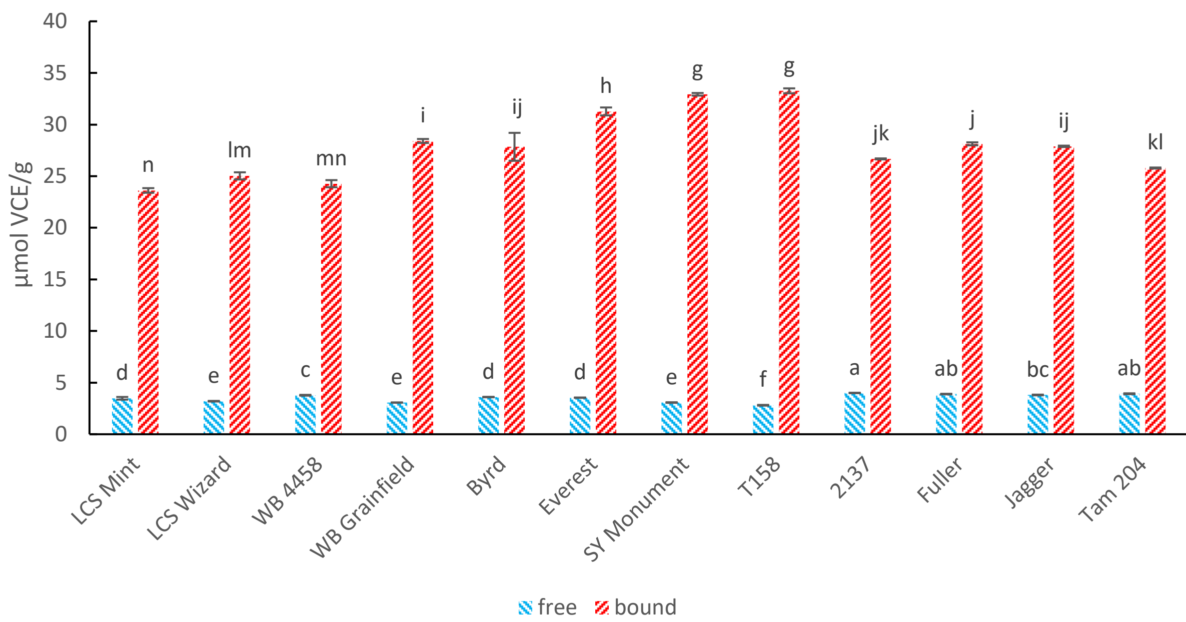

Figure 2-3 DPPH free radical scavenging activity of wheat varieties.

Bars with different letters are significantly different (p<0.05).

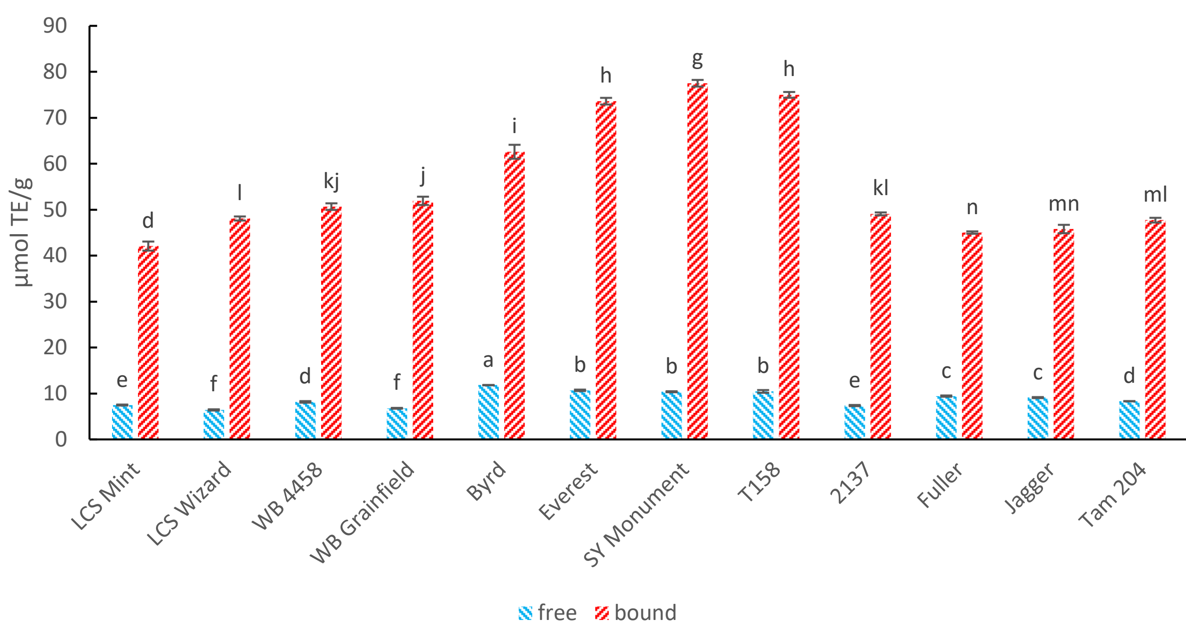

Figure 2-4 ABTS radical scavenging activity of wheat varieties.

Bars with different letters are significantly different ($p<0.05$).

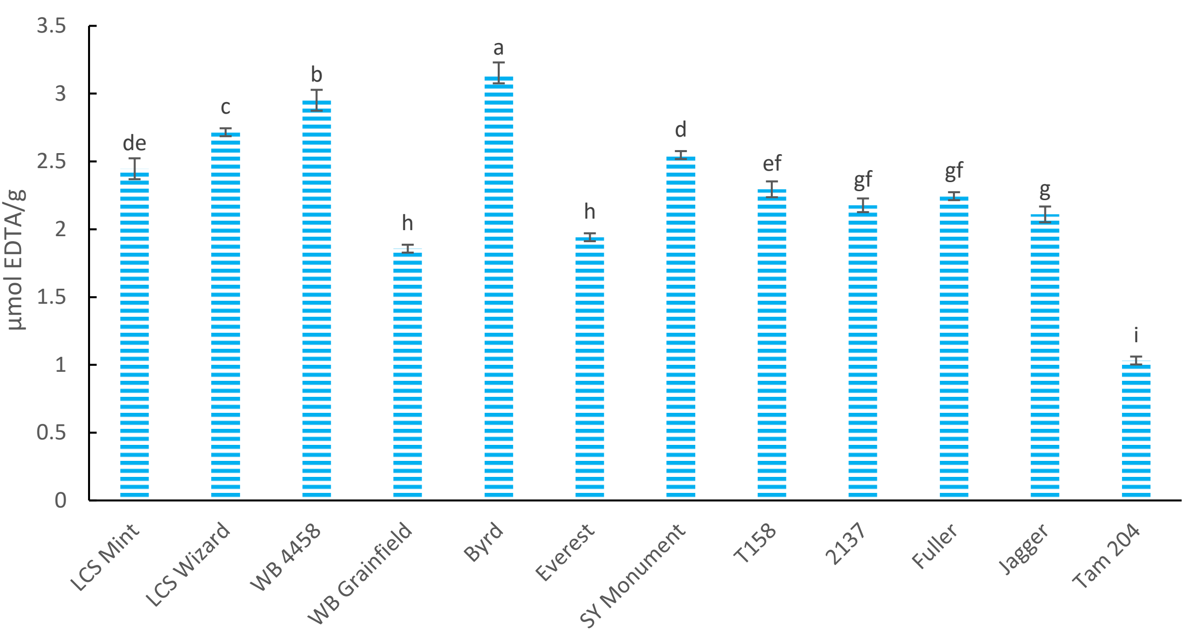

Figure 2-5 Metal chelating activity of wheat activity.

Bars with different letters are significantly different ($p<0.05$)

Chapter 3 - Effects of environment, nitrogen, and sulfur on total phenolic content and phenolic acid composition of winter wheat grain*

Abstract

Health benefits of whole wheat are partially attributed to phenolic compounds. This study reports the effects of harvest year (Y), nitrogen (N) and sulfur (S) fertilization, and wheat variety (V) on total phenolic content (TPC) and phenolic acid composition of wheat grains. The year effect was significant for TPC and all phenolic acids except for syringic acid. The TPC and phenolic acid composition significantly differed among the varieties, except for vanillic acid concentration. Increased nitrogen fertilizer led to increased production of *trans*-ferulic acid, and sulfur application affected the response to nitrogen fertilizer application. Varieties also differed in the response of phenolic acid concentration and composition to sulfur application. To our knowledge, few studies report the effects of nitrogen, sulfur, variety, harvest year, and their interactions on phenolic profiles of hard red winter wheat grains. These results will benefit future wheat production practices that aim to produce wheat grains with enriched natural antioxidants.

Key words: Hard red winter wheat; field management; nitrogen fertilization; sulfur fertilization; year effect; wheat phenolics; antioxidants

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3.1. Introduction

Wheat is among the most important crops for humanity (Shewry, 2009). Genotype, environment, and fertilization are the major determinants of wheat yield and quality (Souza et al., 2004). Generally, wheat quality is determined by protein content, milling performance, dough rheology, and baking properties (Branlard et al., 2001). In recent years, driven by a desire for a healthier diet, potential health benefits of wheat grains have become significant quality parameters. Health benefits of whole wheat are partially attributed to phenolic compounds such as phenolic acids, flavonoids, and phenolic lipids (Liu, 2007). *Trans*-ferulic acid is the predominant phenolic compounds found in wheat (Adom et al., 2003). Ferulic acid in grasses is mostly linked to cell wall arabinoxylans and oligosaccharides through ester bonds (Smith and Hartley, 1983), and can also link to lignin through ether bonds (Scalbert et al., 1985). Vaidyanathan and Bunzel (2012) proposed a classification of ferulic acid in cereal products into free ferulic acid (FFA), ferulic acid ester-linked to mono- and/or oligosaccharides (OF), ester-linked to soluble polysaccharides (SPF), and ester-linked to insoluble polysaccharides (IPF). Isomers of di-ferulic acids (DFA) have also been identified and characterized using GLC-MS (Bunzel et al., 2001), HPLC-UV (Dobberstein and Bunzel, 2010) and HPLC-MS techniques (Gong et al., 2019). Quantification protocol of DFA has been established previously (Jilek and Bunzel, 2013) but the analytical standards are not yet commercially available. Other simple phenolic acids such as 4-hydroxybenzoic acid, vanillic acid, syringic acid, *para*-coumaric acid, and sinapic acid were also detected and quantified in many studies (Fernandez-Orozco et al., 2010; Moore et al., 2005; Tian et al., 2019). Wheat genotypes were reported to affect phenolic acid content and in *vitro* antioxidant capacity of whole wheat flour (Li et al., 2008). Effect of growth year on wheat

phenolics and the year (Y) x variety (V) interactions have been also described in previous studies (Barański et al., 2020; Fernandez-Orozco et al., 2010; Gasztonyi et al., 2011).

Some studies also have reported the effect of fertilizer applications on wheat phenolics and in *vitro* antioxidant capacities (Ma et al., 2015; Stumpf et al., 2015). However, the results of these studies were inconsistent. For example, Ma et al. (2015) found that increased nitrogen application increased soluble total phenolic content (TPC), while Stumpf et al. (2019) showed that soluble TPC was not significantly affected by increasing N application. Therefore, further studies are necessary to understand the effect of nitrogen on TPC. Sulfur application positively impacted carotenoid content of some durum wheat varieties (Fratianni et al., 2013). However, the effect of sulfur fertilizer on phenolic acids, which are major phytochemicals in whole wheat, has not been investigated. Furthermore, little information is available about the interactions of S and N fertilizers and wheat genotypes on these nutraceutical attributes. It is unclear whether different wheat varieties would respond similarly to fertilizer applications in terms of natural antioxidant synthesis and accumulation. This information is becoming important for wheat production as increasing attention has been given to the nutraceutical values of whole wheat products. Synergies of fertility management and variety selection may be critical for production of wheat grains with both enriched nutrient content and desired end-use properties.

The objective of this study was to understand the effect of nitrogen fertilization (N), sulfur fertilization (S), wheat variety, year, as well as their interactions on phytochemical content and antioxidant activities of hard red winter wheat. Total phenolic content (TPC) and phenolic acid composition of four hard red winter wheat varieties were analyzed. To our knowledge, this is the

first study that reports the effects of sulfur application and sulfur x nitrogen, sulfur x variety interactions on phenolic antioxidants of wheat grains. These results will benefit future wheat production and field management practices that aim to produce wheat grains with enriched natural antioxidants. The information also provides new perspectives for the study of plant metabolism responses to fertilizer usage and environmental factors.

3.2. Materials and methods

3.2.1. Field experiment

The experiment was set up in a 3 X 2 X 4 factorial design with four replicates in two years (2017 and 2018). Four wheat genotypes (2137, Everest, Fuller, and Jagger) were chosen based on nitrogen use efficiency and nitrogen uptake. For each genotype, there were 3 nitrogen levels (56, 101 and 146 kg ha^{-1} applied as urea) and 2 sulfur levels (0 and 22 kg ha^{-1} as ammonium sulfate) with four replications. Urea application was adjusted for all S treatments to balance the N rates. The experiment was conducted at the NRCS-Plant Materials Center in Manhattan, Kansas on a Belvue silt loam that was sulfur-deficient and N-deficient based on soil analysis and confirmed by yield response. Complete details about the experimental designs and grain quality analysis were reported in a previous publication(Wilson et al., 2020). After harvest, wheat kernels were kept in a refrigerator and milled to fine whole flour using a Udy mill (2010-030 mill, UDY, Ft. Collins, CO, USA) with a 1 mm stainless steel screen before analysis.

3.2.2. Reagents and chemicals

Standard phenolic acids (4-hydroxybenzoic acid, vanillic acid, syringic acid, *para*-coumaric acid, *trans*-ferulic acid, sinapic acid, and gallic acid) and Folin-Ciocalteu reagent were purchased from

Sigma Aldrich (St. Louis, MO, USA). LC-MS grade solvents and other general chemicals were purchased from Fisher Scientific (Waltham, MA, USA). UPLC sample filter (0.22 μm) was purchased from Millipore Sigma (Burlington, MA, USA).

3.2.3. Extraction of wheat phenolics

Phenolic compounds in the whole flour were extracted according to a previous protocol(Tian et al., 2020b). Briefly, whole flour (1g) was extracted with 10 mL ethanol (80%, v/v) for 20 min. The mixture was hydrolyzed with 5 mL NaOH (6 M) for 3 hours under nitrogen protection in dark conditions. The mixture was acidified to pH 2 with concentrated HCl and then extracted 3 times with ethyl acetate. The extracts were pooled and evaporated using a rotary evaporator, which was then reconstituted to a final volume of 3 mL using LC-MS grade methanol.

3.2.4. Total phenolic content analysis

Total phenolic content (TPC) was determined following a previous method (Tian & Li, n.d.). The extract (0.1 mL) was diluted with 7.9 mL deionized (DI) water and mixed with 0.5 mL 2 N Folin- Ciocalteu reagent for 2 min. Then, 1.5 mL 20% Na_2CO_3 was added to the mixture. Absorbance of the final mixture was determined at 765 nm after 2 hours, and TPC was quantified using gallic acid as an external standard and expressed as mg gallic acid equivalence (GAE)/g sample.

3.2.5. Analysis of phenolic acid composition

A UPLC-DAD-ESI-Q-TOF-MS/MS system from Waters Corporation (Milford, MA, USA) was used for identification and quantification of phenolic acids. Mobile phase A was water

containing 0.1% formic acid, and mobile phase B was acetonitrile containing 0.1% formic acid. The gradient protocol and detector setups were reported in our previous study (Tian et al., 2021c). Simple phenolic acids were identified and quantified according to analytical standards. Due to a lack of analytical standards, isomers of DFA were identified according to the MS^E spectra and relative retention times reported in previous studies using similar gradient protocols (Dobberstein & Bunzel, 2010; E. S. Gong et al., 2019) and quantitively estimated according to the external standard curve of *trans*-ferulic acid.

3.2.6. Data analysis

Data analysis was performed using JMP 15 software from SAS institute (Cary, NC, USA). Nitrogen (N), sulfur (S), variety (V), year (Y), and their interactions were considered as fixed effects. Replicates from 4 plots were considered as random effects. The Standard Least Squares personality package within the Fit Model platform was used. The Emphasis option was set to Effect Screening and the Method option was set to restricted maximum likelihood (REML). Effects were considered to be significant at a confidence level of $p < 0.05$. Least square (LS) mean value, instead of simple average value, was used when discussing the factor effect. Tukey’s honestly significant difference (HSD) test was used to evaluate treatment differences. Principal component analysis (PCA) and correlation analysis were performed using Unscrambler 11 software from CAMO Analytics (Oslo, Norway).

3.3. Results and discussions

3.3.1. Raw experimental data

Monomeric phenolic acids (4-hydroxybenzoic acid, vanillic acid, syringic acid, *para*-coumaric acid, *trans*-ferulic acid, sinapic acid and *cis*-ferulic acid) and di-ferulic acid (DFA) isomers (8-8 DFA, 5-5 DFA, 8-5 DFA, 8-O-4 DFA and 8-5'Benzofuran DFA) were identified by LC-MS analysis. Due to the complexity nature of DFA isomers (Bunzel, 2010; Waterstraat and Bunzel, 2019), the estimated concentrations of all five DFA isomers were added together and labelled as "DFA Sum" in this study. Concentration of *trans*-ferulic acid ranged from 575.0 to 875.7 µg/g. A recent review paper summarized that range of total concentration of *trans*-ferulic acid was from 269.2 to 744.7 µg/g whole flour (Liu et al., 2020), which was consistent but to some extent lower than our results. This might be due to effect of sulfur rate in the soil as discussed in Section 3.4 and 3.5. Total concentration of other phenolic acids was generally less than 50 µg/g flour. This range was consistent with previous studies (Liu et al., 2020; Ward et al., 2008).

3.3.2. Summary of ANOVA

Our preliminary analysis suggested that the effect of year interactions (i.e. year x nitrogen, year x sulfur, year x variety) on total phenolic content and *trans*-ferulic acid were mostly non-significant (**Table 3-1**). Therefore, the year interactions with treatment were not included during formal analysis. Results of analysis of variance (ANOVA) are summarized in Nitrogen x sulfur x variety interaction was not significant for total phenolic content and concentration of phenolic acids. Nitrogen x sulfur interaction was significant for *trans*-ferulic acid. Sulfur x variety interaction was significant for TPC, *trans*-ferulic acid, and sum of di-ferulic acid (DFA) isomers. Year effect was significant for TPC and all phenolic acids except for syringic acid. Nitrogen

application rate was significant for 4-hydroxybenzoic acid, sinapic acid, and *cis*-ferulic acid. Sulfur application rate was significant for the concentration of 4-hydroxybenzoic acid, *cis*-ferulic acid, and sum of DFA isomers. Wheat variety was significant for the concentration of 4-hydroxybenzoic acid, syringic acid, *para*-coumaric acid, sinapic acid, *cis*-ferulic acid and DFA isomers.

3.3.3. Effect of harvest year

In 2018, the weather was hot and dry during the grain filling period (**Figure 3-1**) (Wilson et al., 2020). Such weather conditions may benefit accumulation of phenolic compounds. Comparison of TPC and phenolic acids between 2017 and 2018 is summarized in **Table 3-2**. LS mean TPC of 2018 samples was significantly higher than that of the 2017 samples. The year effect was also significant for *trans*-ferulic acid. *Trans*-ferulic acid is the predominant phenolic compound in whole wheat and a major contributor to TPC. As shown in **Table 3-2**, the year effect was significant for most phenolic acids except for syringic acid. The concentration of 4-hydroxybenzoic acid, vanillic acid, *para*-coumaric acid, *cis*-ferulic acid, and isomers of di-ferulic acids (DFA) significantly increased from 2017 to 2018. However, the concentration of sinapic acid decreased significantly from 2017 (35.3 μg/g) to 2018 (30.0 μg/g).

Generally, our results agreed with previous studies that demonstrated that harsh weather conditions led to increased concentrations of phenolic compounds (Gasztonyi et al., 2011; Ma et al., 2014; Shewry et al., 2010). Several studies found that wheat phenolics significantly decreased during the maturity process (Barros Santos et al., 2019; Kim and Kim, 2016; Özkaya et al., 2018). Heat stress in 2018 may have interfered with the normal maturation process (De

Leonardis et al., 2015); therefore, relatively more phenolics remained in the mature wheat kernels. We also observed that syringic acid was less sensitive to year variance, and sinapic acid concentration decreased significantly under the harsh conditions in 2018. This result suggested that syringic acid and sinapic acid may arise from different metabolic pathways and may merit further investigation by plant physiologists.

3.3.4. Sulfur x variety interaction

Sulfur x variety interaction was significant for TPC, *trans*-ferulic acid, and DFA isomers (**Table 3-2**). Effect of sulfur x variety interaction on TPC is shown in **Figure 3-2(a)**. Sulfur significantly increased the TPC in 2137, but not in the other varieties. The small, but insignificant, increase of TPC observed for Everest and Jagger may have contributed to the significant overall main effect observed for the increased TPC with S application (**Table 3-2**). The general tendency was that sulfur application increased TPC for those varieties with lower TPC in the absence of sulfur. For example, in the absence of sulfur fertilization, TPC for Fuller was greater than all other genotypes; however, with sulfur application, there was no significant difference in TPC between Fuller and Everest. It seems that the TPC response of Fuller is less sensitive to sulfur deficiency, whereas the TPC response of 2137 is more sensitive to sulfur deficiency. Fuller variety can retain high TPC regardless of fertilization. It can be concluded that the effect of sulfur application on TPC was highly dependent on wheat variety, as also demonstrated through the significant sulfur x variety interaction effect on TPC ($p < 0.05$) based on ANOVA (**Table 3-2**).

The effect of sulfur x variety interaction on *trans*-ferulic acid is plotted in **Figure 3-2(b)**. Unlike TPC where application of sulfur had a positive effect, the application of sulfur fertilizer

significantly decreased the concentration of *trans*-ferulic acid for Fuller (from 781.4 to 708.3 μg/g) and Everest (from 753.7 to 714.2 μg/g). For Jagger and 2137, the decrease was not significant ($p>0.05$) though a similar trend was observed. It seemed that wheat varieties with higher content of *trans*-ferulic acid were more likely to be affected by sulfur application while other varieties were more resistant to the sulfur application.

Sulfur x variety interaction was also significant for DFA isomers. The interaction effect is plotted in **Figure 3-2(c)**. The interaction pattern was more complicated for DFA isomers. The effect of sulfur application was highly dependent on wheat variety. For Everest, application of sulfur fertilizer led to decreased concentration for most DFA isomers. For Jagger, the sulfur application significantly increased amount of DFA isomers. Similarly, for 2137 and Fuller, mean values of DFA isomers for S= 22 (sulfur application at 22 kg ha^{-1} as ammonium sulfate) groups were higher than that for S= 0 (no ammonium sulfate application) groups though these differences were not statistically significant.

3.3.5. Sulfur x nitrogen interaction

The response of *trans*-ferulic acid concentration to increasing nitrogen fertility varied depending on sulfur application rate. The interaction effect from sulfur application is plotted in **Figure 3-3**. Where sulfur was not applied, increased nitrogen application led to increased concentration of *trans*-ferulic acid. However, when sulfur was applied, increased nitrogen rate did not have apparent effects on *trans*-ferulic acid concentration.

3.3.6. Principal component analysis

Principal component analysis (PCA) provided visualizations of main effects and effects of traits (TPC and each phenolic acid) in the differentiation and classification of whole flours. Principal component- 1 (PC- 1) explained 35% of the total variation, and PC- 2 explained 23% of the total variation. Whole flours were well separated by harvest year along PC-1 axis (**Figure 3-4(a)**), which confirmed the significance of year effect. DFA isomers and total phenolic content (TPC) are the major contributors to PC-1 (Figure **3-4(b)**), which suggested that weather conditions of different years had stronger effect on DFAs compared to other simple phenolic acids. PCA plots by wheat variety (**Figure 3-5(a)**), nitrogen level (**Figure 3-5(b)**), and sulfur level (**Figure 3-5(c)**) are provided. The varieties 2137 and Everest were mainly in the positive direction of PC-2, while Fuller and Jagger were mainly in the negative direction of PC-2. *Cis*-ferulic acid and sinapic acid were responsible for this separation along PC-2 axis. Analysis of original data also found that 2137 and Everest had higher amount of *cis*-ferulic acid than Fuller and Jagger, while varieties Fuller and Jagger had higher amount of sinapic acid (**Table 3-4**). This analysis suggested that though *trans*-ferulic acid was the major phenolic acid, other monomeric phenolic acids can be important indicators for variances among wheat genotypes. From the PCAs, it is clear that there is correlation among the response variables (**Table 3-5**).

3.3.7. Net yield of phenolic compounds per hectare

The analysis and discussion above are based on the concentration of phenolics in a whole wheat flour. However, the net yield of phenolics may also be an important production consideration. In our study, grain yield for 2017 and 2018 was 2783 kg/ha and 1580 kg/ha, respectively (Wilson et al., 2020); TPC for 2017 and 2018 was 1.79 mg GAE/g and 1.92 mg GAE/g, respectively.

Though 2018 wheats were relatively more concentrated with phenolic compounds, 2017 wheats had much higher net phenolic production. The converted yield for 2017 wheats was 4982 g GAE/ha, while that of 2018 wheats was 3034 g GAE/ha. Similarly, sulfur application decreased the amount of *trans*-ferulic acid by 5% (709.28 µg/g to 674.46 µg/g flour), but it significantly increased the grain yield by over 50% (Wilson et al., 2020). and therefore increased net production of *trans*-ferulic acid per hectare**.** Future studies are advised to consider the net yield in addition to the concentration of phenolics, though the concentration of phenolics is more important solely from a health standpoint.

3.4. Conclusions

Year, sulfur fertilization, and wheat variety significantly influenced the total phenolic content and phenolic acid composition of wheat grains. Significant sulfur x variety interactions were observed for TPC, *trans*-ferulic acid, and DFA isomers. Increased nitrogen application led to enhanced production of *trans*-ferulic acid. Sulfur application increased TPC, concentrations of 4-hydroxybenzoic acid, *p*-coumaric acid but decreased concentration of *trans*-ferulic acid. Though nitrogen and sulfur applications had some effect on wheat phytochemicals, environment (e.g., year) and variety differences dominated the analysis of variance. Although favorable weather conditions (e.g., 2017 vs. 2018) decreased phytochemical concentration in whole wheat flour, the total phenolic production per hectare was still much higher under favorable weather conditions and optimized fertility.

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Conflict of interest

The authors declare that there is no known conflict of interests.

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Figures and tables

Table 3-1 Original results of analysis of variance (ANOVA) on total phenolic content (TPC) and *trans*-ferulic acid.

	TPC		***Trans*-ferulic acid**	
Source	**F Ratio**	**Prob > F**	**F Ratio**	**Prob > F**
Year	77.9956	0.0001*	24.6008	0.0026*
Nitrogen	1.0894	0.3675	6.0300	0.0154*
Sulfur	14.0672	0.0015*	44.7478	<.0001*
Sulfur x Nitrogen	0.4407	0.6503	5.2198	0.0163*
Variety	47.8603	<.0001*	233.3770	<.0001*
Variety x Nitrogen	1.7003	0.1278	0.4922	0.8129
Variety x Sulfur	3.7312	0.0135*	14.9980	<.0001*
Variety x Sulfur x Nitrogen	0.7170	0.6367	0.9644	0.4530
Nitrogen x Year	1.2742	0.3149	1.7372	0.2175
Sulfur x Year	2.6888	0.1184	1.9573	0.1788
Sulfur x Nitrogen x Year	1.4410	0.2627	0.5190	0.6038
Variety x Year	0.5181	0.6707	3.3824	0.0209*
Variety x Nitrogen x Year	1.2958	0.2653	1.1486	0.3395
Variety x Sulfur x Year	0.4202	0.7389	1.2122	0.3089
Variety x Sulfur x Nitrogen x Year	0.8370	0.5440	0.6709	0.6733

Table 3-2 Summary of formal analysis of variance results

	TPC	4-HA	VA	SyrA	pCA	tFA	SipA	cFA	DFA Sum
Year	***	***	***	ns	***	**	**	**	***
Nitrogen	ns	*	ns	ns	ns	*	***	***	ns
Sulfur	**	*	ns	ns	***	***	ns	***	ns
Variety	***	***	ns	***	***	***	***	***	***
N x S	ns	ns	ns	ns	ns	*	ns	ns	ns
N x V	ns	ns	ns	ns	*	ns	ns	ns	ns
S x V	*	ns	ns	ns	ns	***	ns	ns	***

***, **, * indicate significance at $p < 0.001$, 0.01, and 0.05, respectively. ns: no significant difference. 4-HA: 4-hydroxybenzoic acid; VA: vanillic acid; SyrA: syringic acid; pCA: *para*-coumaric acid; tFA: *trans*-ferulic acid; SipA: sinapic acid; cFA: *cis*-ferulic acid; DFA: di-ferulic acid.

Table 3-3 Effect of harvest year on TPC and phenolic acid composition of wheat varieties.

	2017	2018
TPC (mg GAE/ g)	1.79b	1.92a
4-Hydroxybenzoic acid (µg/g)	14.7b	19.8a
Vanillic acid(µg/g)	19.0b	27.9a
Syringic acid(µg/g)	8.2a	9.3a
Para-coumaric acid (µg/g)	12.2b	15.6a
Trans-ferulic acid (µg/g)	677.5b	706.2a
Sinapic acid(µg/g)	35.3a	30.0b
Cis-ferulic acid(µg/g)	33.6b	34.7a
DFA Sum(µg/g)	111.2b	133.4a

Within each row, values with no letter in common are considered to be significantly different ($p < 0.05$).

Table 3-4 Average concentration of sinapic acid and *cis*-ferulic acid of wheat varieties.

	sinapic acid (µg/ g)	*cis*-ferulic acid (µg/ g)
2137	24.00	34.64
Everest	22.89	38.09
Fuller	46.14	32.59
Jagger	37.48	31.39

Table 3-5 Correlation table among response variables.

	TPC	4-HA	VA	SyrA	p-CA	t-FA	SipA	c-FA	DFA Sum
TPC	1.00	0.53	0.42	0.27	0.56	0.60	0.20	-0.02	0.62
4-HA	0.53	1.00	0.37	0.32	0.22	0.11	0.49	-0.32	0.41
VA	0.42	0.37	1.00	-0.08	0.42	0.15	-0.17	-0.03	0.26
SyrA	0.27	0.32	-0.08	1.00	0.02	0.22	0.35	-0.11	0.38
p-CA	0.56	0.22	0.42	0.02	1.00	0.55	-0.28	0.14	0.34
t-FA	0.60	0.11	0.15	0.22	0.55	1.00	0.09	0.43	0.36
SipA	0.20	0.49	-0.17	0.35	-0.28	0.09	1.00	-0.44	-0.05
c-FA	-0.02	-0.32	-0.03	-0.11	0.14	0.43	-0.44	1.00	0.14
DFA Sum	0.62	0.41	0.26	0.38	0.34	0.36	-0.05	0.14	1.00

4-HA: 4-hydroxybenzoic acid; VA: vanillic acid; SyrA: syringic acid; pCA: *para*-coumaric acid; tFA: *trans*-ferulic acid; SipA: sinapic acid; cFA: *cis*-ferulic acid; DFA: di-ferulic acid.

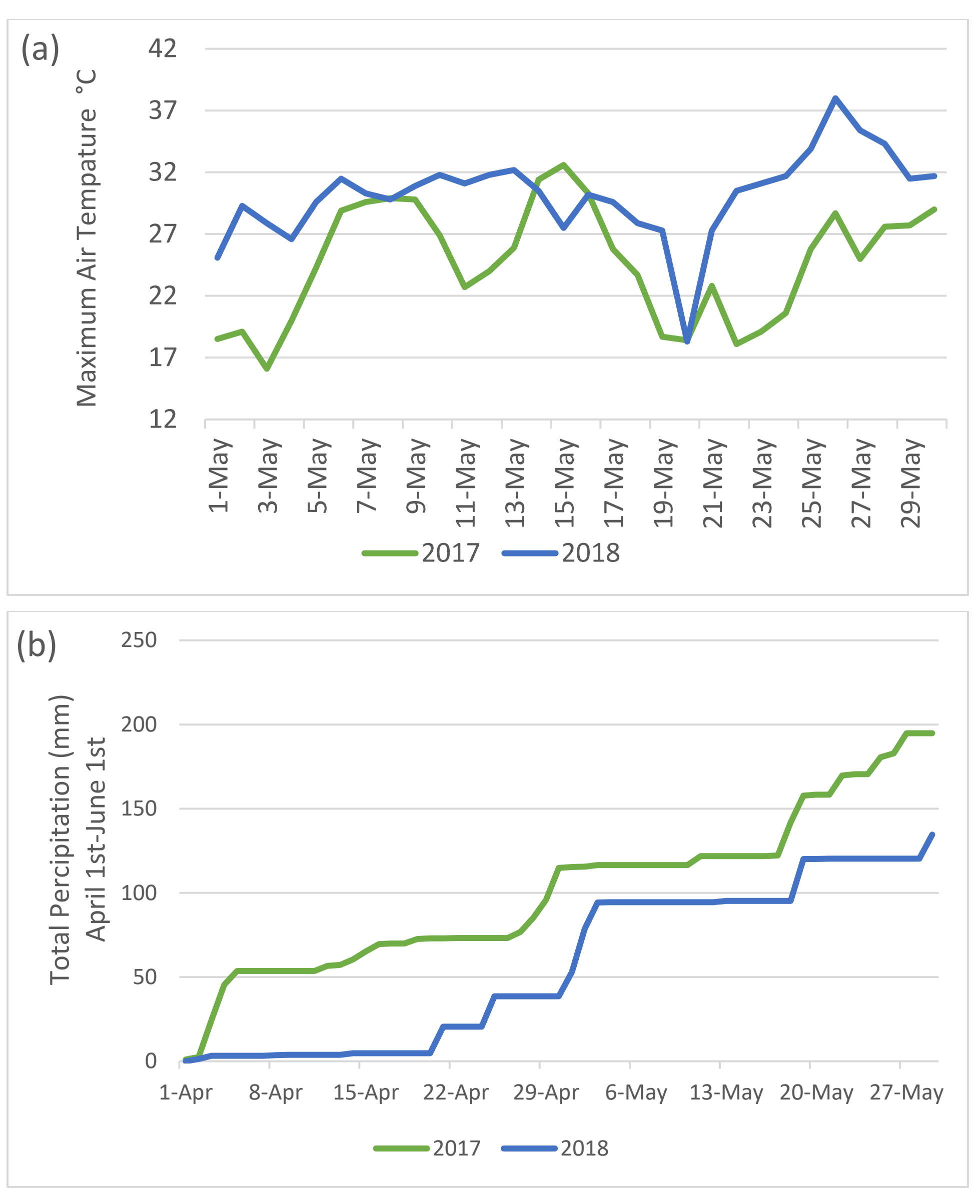
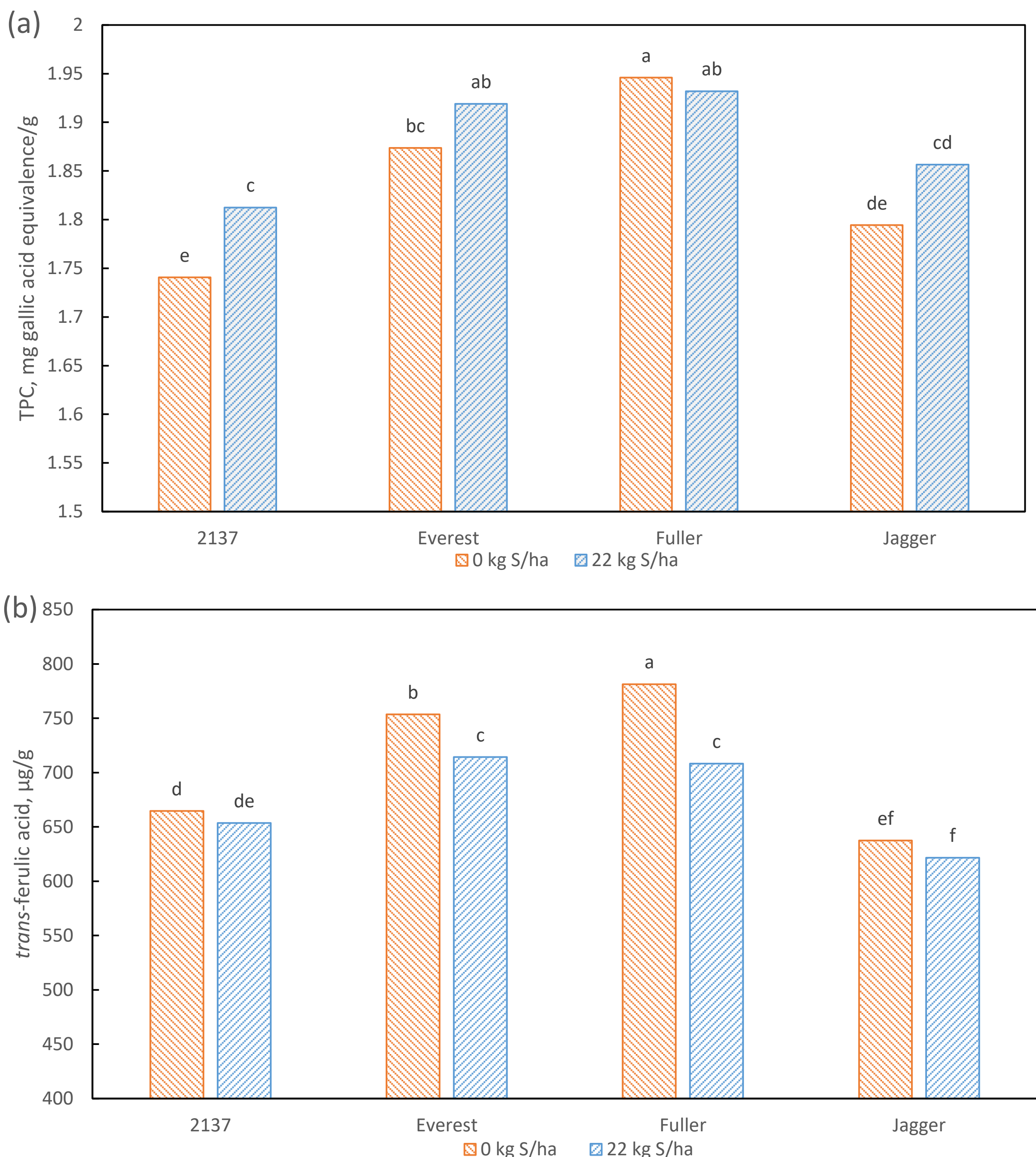


Figure 3-1 (a) Maximum air temperature (°C) for May 2017 and 2018 during grain fill; (b) Cumulative precipitation from April 1 – Jun 1.

Data from Kansas Mesonet (http://mesonet.k-state.edu/). These figures have been presented in Supplementary Document in a previous publication (Wilson, T.L., Guttieri, M.J., Nelson, N.O., Fritz, A. and Tilley, M., 2020. Nitrogen and sulfur effects on hard winter wheat quality and asparagine concentration. Journal of Cereal Science, p.102969).


(a)
TPC, mg gallic acid equivalence/g
2
1.95
1.9
1.85
1.8
1.75
1.7
1.65
1.6
1.55
1.5
e
c
bc
ab
a
ab
de
cd
2137
Everest
Fuller
Jagger
0 kg S/ha
22 kg S/ha
(b)
trans-ferulic acid, µg/g
850
800
750
700
650
600
550
500
450
400
d
de
b
c
a
c
ef
f
2137
Everest
Fuller
Jagger
0 kg S/ha
22 kg S/ha

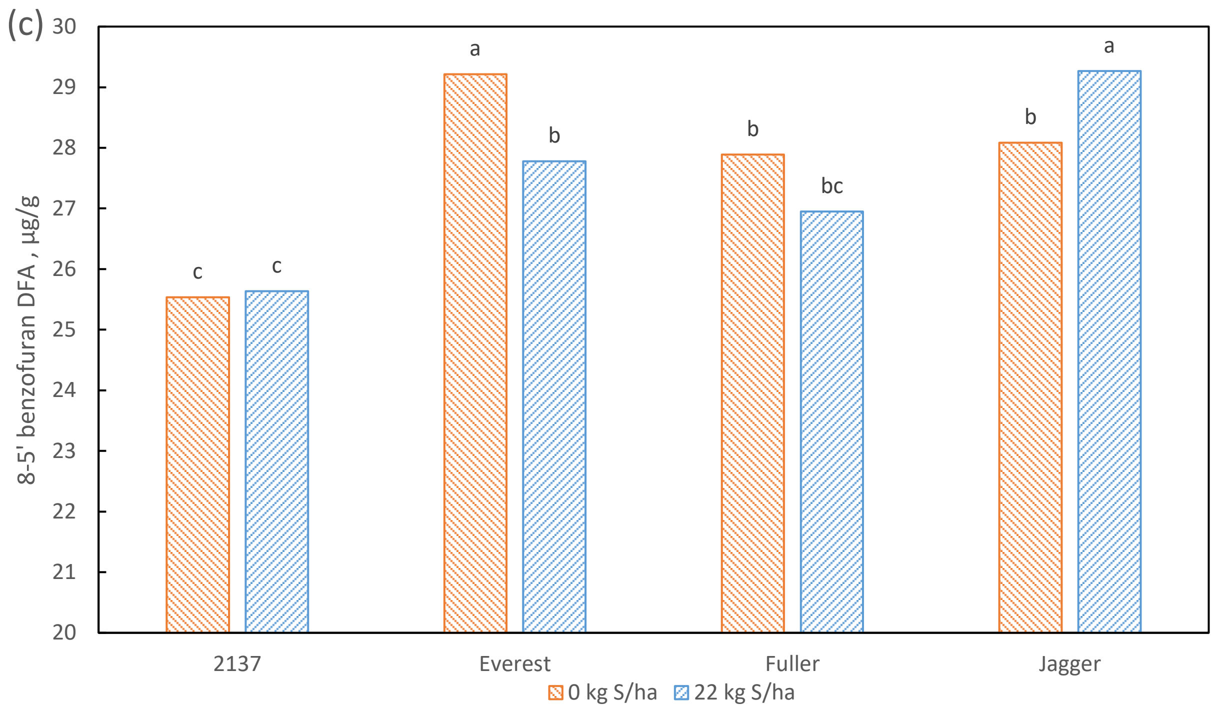


Figure 3-2 (a) Sulfur x variety interaction effect on total phenolic content (TPC); (b) Sulfur x variety interaction effect on *trans*-ferulic acid; (c) Sulfur x variety interaction effect on 8-O-4 benzofuran DFA.

Values with no letter in common in each graph are considered as significantly different ($p < 0.05$).

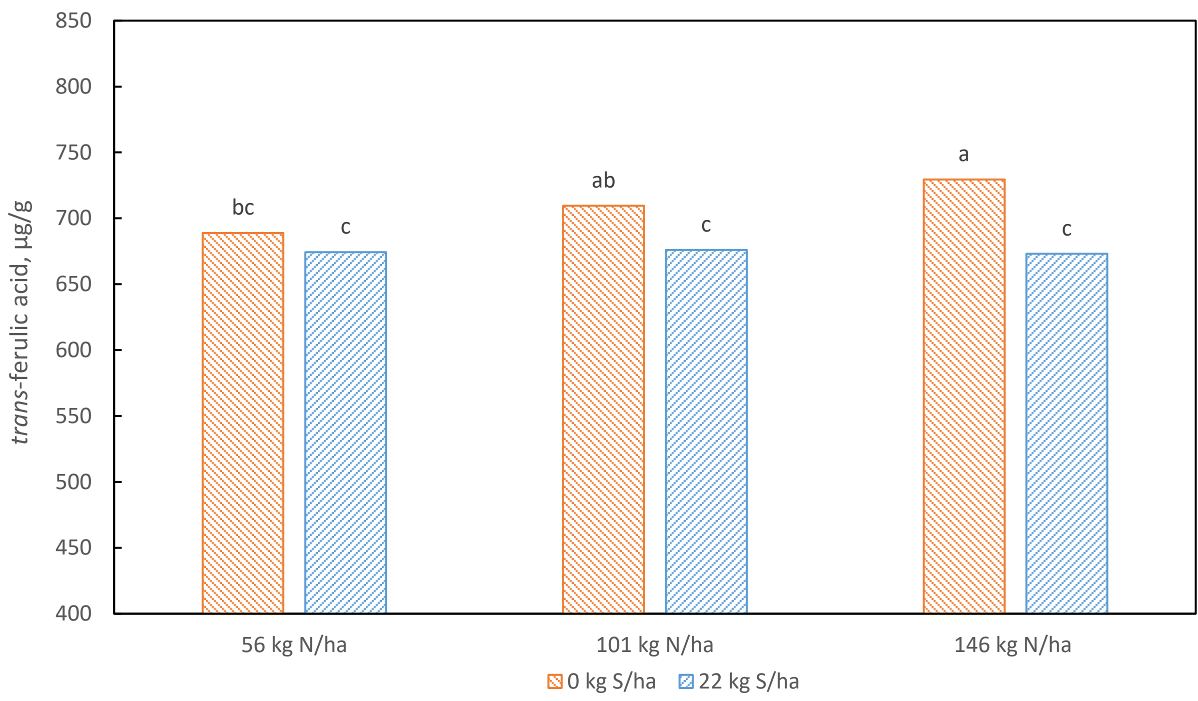


Figure 3-3 Nitrogen x sulfur interaction effect on *trans*-ferulic acid.

Values with no letter in common are considered as significantly different ($p < 0.05$).

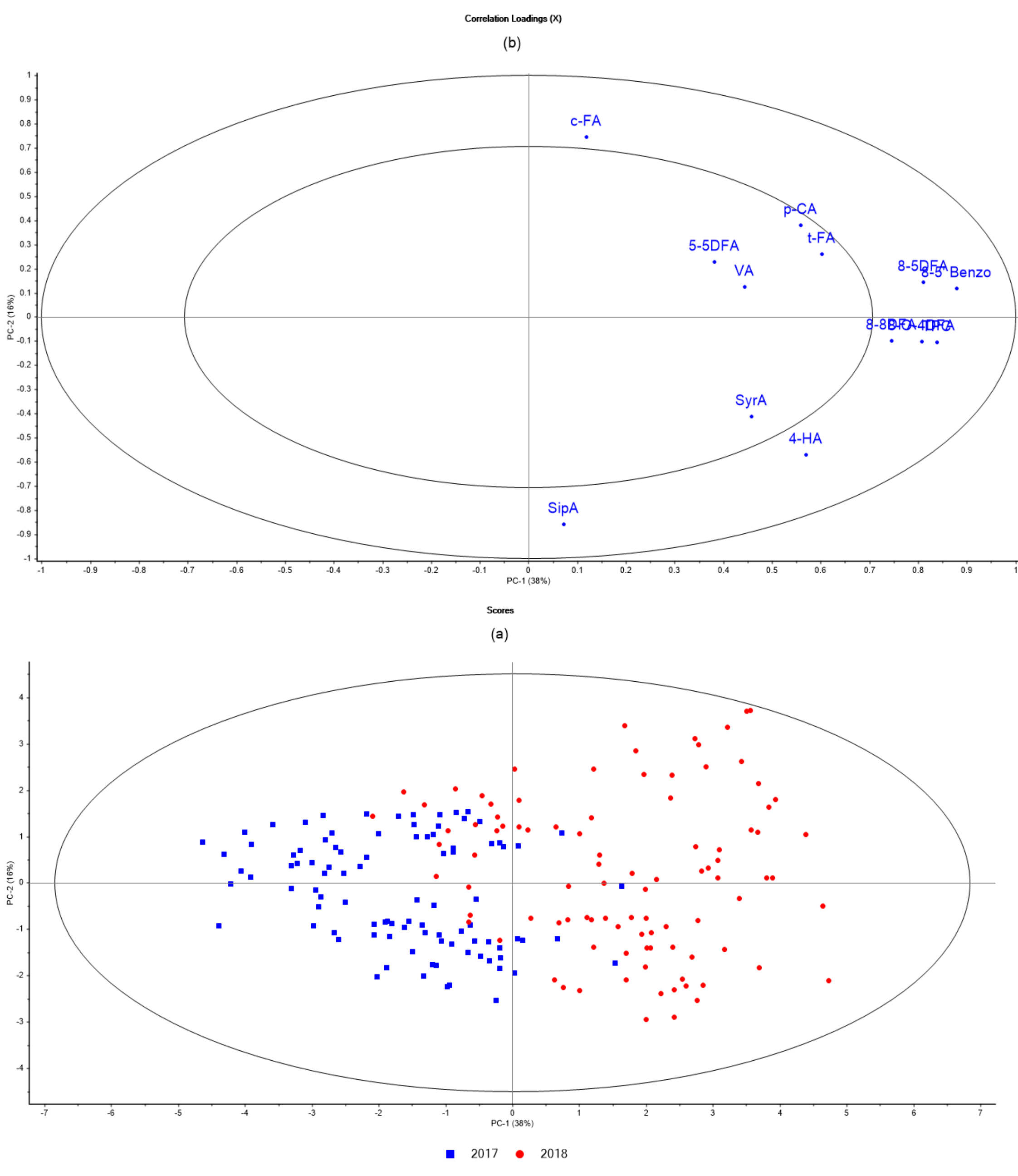
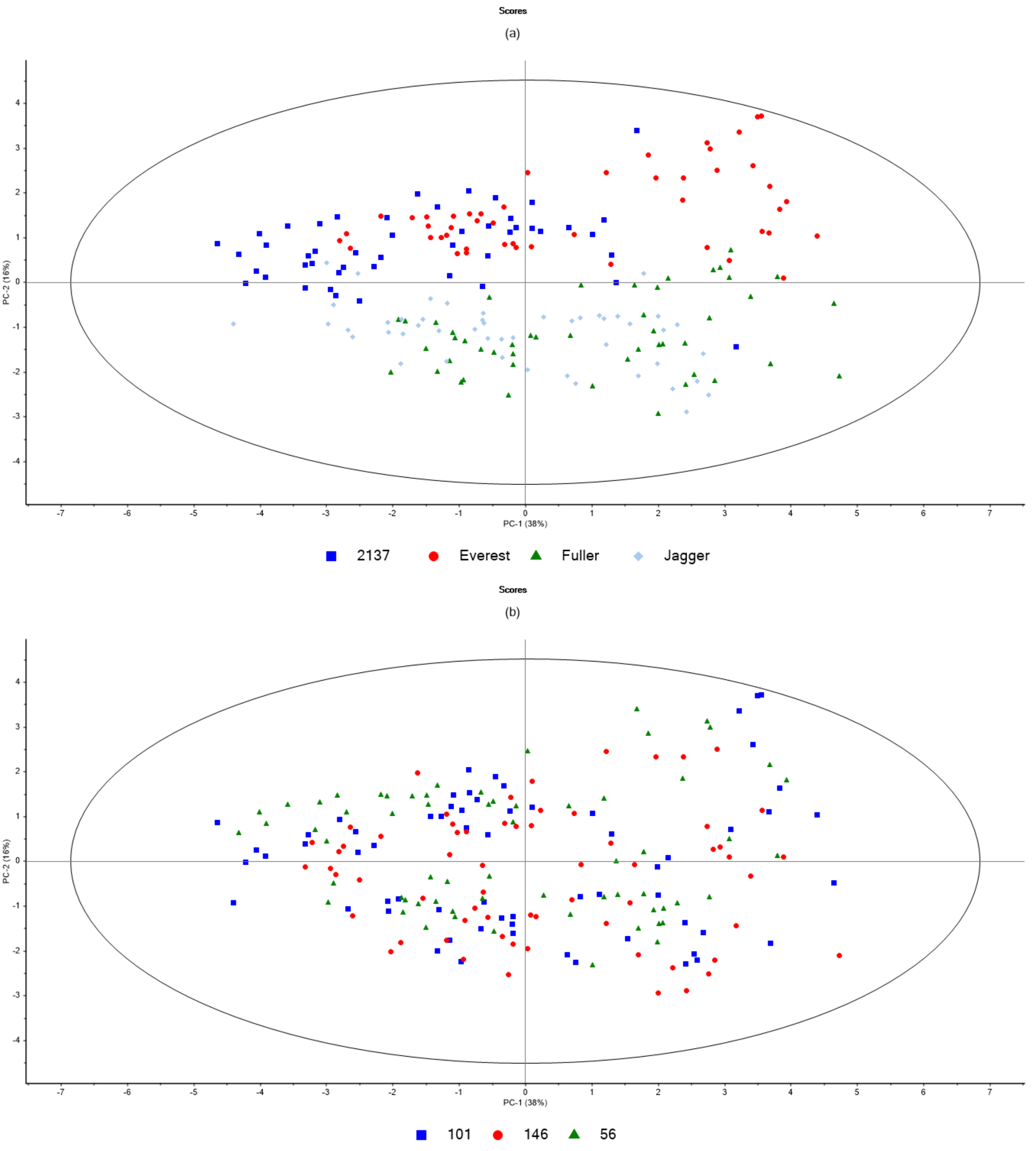


Figure 3-4 Principal component analysis (PCA). (a) Classification of all the measurements (TPC, phenolic acids) based on different harvest years; (b) Correlation loading plot of the PCA showing contribution of TPC and phenolic acids.

4-HA: hydroxybenzoic acid; VA: vanillic acid; SyrA: syringic acid; p- CA: para-coumaric acid;

t-FA: *trans*-ferulic acid; SipA: sinapic acid; c-FA: *cis*-ferulic acid; DFA: di-ferulic acid


Scores
(a)
PC-2 (16%)
PC-1 (38%)
2137
Everest
Fuller
Jagger
Scores
(b)
PC-2 (16%)
PC-1 (38%)
101
146
56

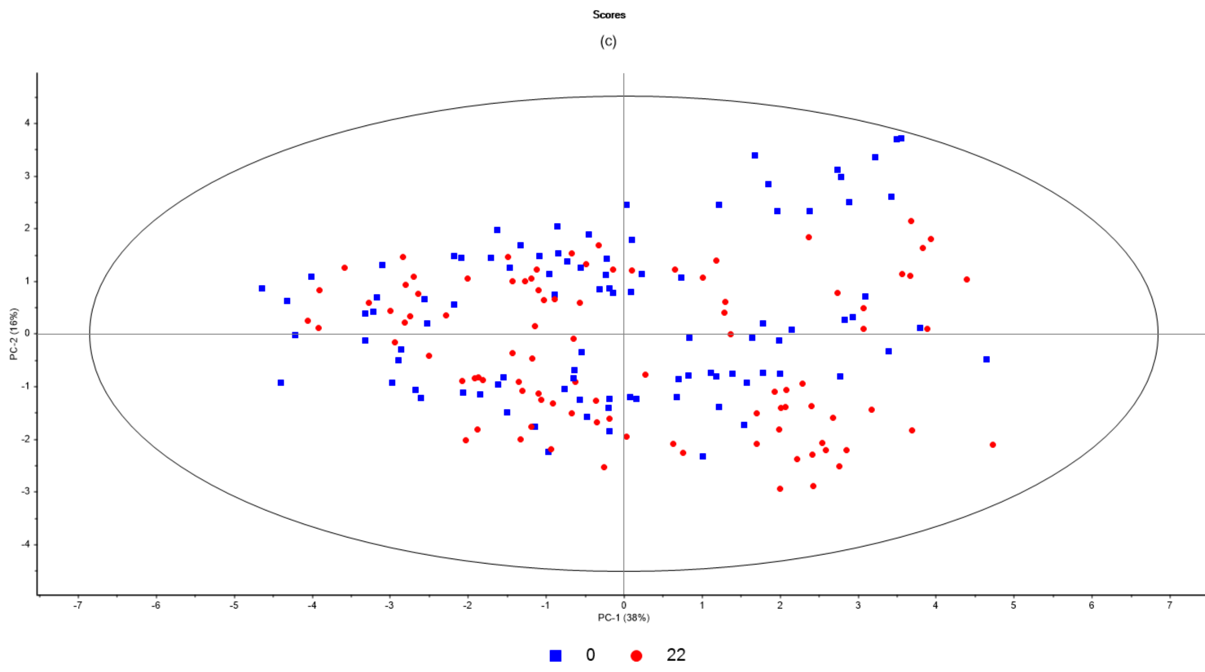


Figure 3-5 (a) PCA analysis on wheat variety; (b) PCA analysis on different nitrogen levels (56, 101 and 146 kg ha-1); (c) PCA analysis on different sulfur levels (0 and 22 kg ha-1).

Chapter 4 - Effect of environment and field management strategies on phenolic profiles of winter wheat*

Abstract

Integrated wheat management strategies are known to affect grain yield and flour end-use properties. However, effect of integrated managements and their interaction with environmental factors on phenolic profiles of wheat have not been reported previously. In this study, we found that year x location x management and year x management x variety interactions were significant for total phenolic content (TPC) of wheat samples. Year x location x management x variety interaction was significant for concentration of *trans*-ferulic acid and several other phenolic acids. Managements with fungicide application (e.g., farmer' s practice and enhanced fertility) may lead to decreased accumulation of phenolic compounds, especially for WB5558 which is relatively more susceptible to fungi infection. However, this effect was also interacted by harvest year and growing locations. Further, higher original nitrogen level in the soil may positively affect TPC and phenolic acid concentrations. In summary, this is the first study that reported interactions of wheat management strategies, wheat variety, and environmental factors on phenolic profile of winter wheat.

Key Words: integrated wheat managements; wheat phenolic profile; environment interaction; fungicide application; fertilizer application

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* This chapter has been under review for publication. Tian, W., Jaenisch, B., Gui, Y., Hu. R., Chen, G., Lollato, R., Li, Y. Effect of environment and field management strategies on phenolic acid profiles of hard red winter wheat genotype.

4.1. Introduction

Wheat (*Triticum* spp.) is a worldwide staple food (Godfray et al., 2010). World trade of wheat is greater than that of all other crops combined (Giraldo et al., 2019). Wheat protein content is essential for its end-use properties such as bread-making properties(Asseng et al., 2019). Traditionally, wheat quality is majorly determined by grain yield and end-use properties. Proper wheat management strategies can possibly enhance yields and end-use quality of wheat grains. Common strategies include enhanced fertility, increased foliar protection, and application of micronutrients (Munaro et al., 2020).

Consumption of whole grain products has been associated with many health benefits such as reduced risk of chronic diseases(Liu, 2007). The health benefits can partially be attributed to wheat phytochemicals that mainly exist in wheat brans (Jonnalagadda et al., 2011). Phenolic acids are among the major phytochemicals in wheat brans. Phenolic acids exist in soluble-free, soluble-conjugated and insoluble-bound forms(Adom et al., 2003; Tian and Li, 2018). Insoluble-bound phenolics may survive the upper gastrointestinal digestion and reach the colon where they are released by gut microbiota fermentation and exhibit site-specific benefit for the colon health(Liu, 2007). Due to consumers' desire for healthier food ingredients, concentration of phenolic compounds has become another aspect with increasing interest for wheat quality evaluation. Many previous literatures have reported the effect of environmental factors and wheat genotypes on wheat phenolic profile and antioxidant activities (Liu et al., 2020; Shewry et al., 2010). Several studies also discussed effect of fertilizer usages on wheat phenolic profiles (Gasztonyi et al., 2011; Ma et al., 2014; Stumpf et al., 2015). However, there are many other

farming practices and strategies besides fertilizer application during wheat growth. A wheat management strategy is an integration of choices including but not limited to till and no-till, application of fungicide, application of micronutrient, and use of in-furrow starter fertilizer (Munaro et al., 2020). Effects of integrated management strategies, instead of a single treatment such as fertilizer usage, on wheat phenolics have not been reported in previous studies. Furthermore, interaction effect of environment factors and management strategies should be investigated so that farmer can properly adjust management strategies based on weather conditions and market preference. In this study, we reported effect of year, farm location, and common wheat management strategies on phenolic profile of wheat. Management strategies consisted of a general farmer practice (FP), enhanced fertility (EF), economical intensification (EI), increased foliar protection (IFP), and water-limited yield potential (Yw). We hypothesized that wheat management strategies can affect phenolic acid composition of wheat and this effect is also interacted with harvest year and growing location. To our knowledge, this is the first study that reported effects of environment and integrated managements strategies on phenolic profiles of wheat.

4.2. Materials and methods

4.2.1. Field experiments

Experiments were conducted in a split-plot design in a complete factorial treatment structure with whole plots arranged as randomized complete block design and subplots completely randomized within whole plot at three locations during the growing seasons of 2017-2018 and 2018-2019 (B. Jaenisch & Lollato, 2019). Management strategies consisted of a general farmer practice (FP), enhanced fertility (EF), economical intensification (EI), increased foliar protection

(IFP), and water-limited yield potential (Yw). Two winter wheat genotypes (WB4458 and WB-Grainfiled) were selected for this study. Locations included Belleville in north central Kansas (moderately well-drained Crete silt loam, 0 to 1 percent slopes), Hutchinson in south central Kansas (well-drained Ost loam, 0 to 1 percent slopes), and Leoti in western Kansas (well-drained Richfield silt loam, 0 to 1 percent slopes). Wheat was grown in all locations under rainfed conditions, and these locations were chosen to represent the variability of the environment throughout Kansas (Lollato, Bavia, et al., 2020). The FP consisted of a seeding rate of 2.7 million seeds ha^{-1}, and N application was for a yield goal representative the ten-year county wheat grain yield average (about 2.4 Mg ha^{-1}). Enhanced fertility consisted of 112 kg of MESZ ha^{-1} placed in-furrow with the seed (which provided 14 kg N ha^{-1}, 45 kg P_2O_5 ha^{-1}, 11 kg S ha^{-1}, and 1 kg Zn ha^{-1}) and additional N rate for a 6.7 Mg ha^{-1} yield goal applied at Feekes growth stage (GS) 3-4 (Large, 1954). Economical intensification consisted of EH plus one fungicide application at Feekes GS10.5. Increased foliar protection consisted of EH plus two fungicide applications at Feekes GS6 and GS10.5. Water-limited yield potential consisted of IFP plus a micronutrient application at Feekes GS6. The entire plots were harvested at physiological maturity using a XP 8 Massey Ferguson small plot combine. After harvest, wheat grains were milled to separate endosperm and bran fractions according to AACC Approved Method 26-50.01. Wheat brans were kept at 4 °C until uses for phenolic extraction and analysis.

4.2.2. Reagents and chemicals

Folin- Ciocalteu reagent and standard phenolic acids (4-hydroxybenzoic acid, vanillic acid, syringic acid, *para*-coumaric acid, *trans*-ferulic acid, sinapic acid, gallic acid) were purchased from MilliporeSigma (St. Louis, MO, USA). Other general chemicals and LC-MS mobile phases

(water with 0.1% formic acid and acetonitrile with 0.1% formic acid) were purchased from Thermo Fisher Scientific (Waltham, MA, USA)

4.2.3. Extraction of phenolics from wheat bran

Phenolic compounds in wheat bran samples were extracted according to a method reported previously (Tian et al., 2020a) with minor modifications. In brief, one gram of the bran was first mixed in 10 mL of 80% ethanol for 20 min. The mixture was then hydrolyzed with 5 mL of 6 M NaOH for 4 hours under nitrogen protection in dark condition. The sample was then acidified with concentrated HCl to pH 2 and extracted five times with ethyl acetate. The combined organic phase was evaporated to dryness and reconstituted to a final volume of 3 mL with LC-MS grade methanol. The phenolic extract was filtered through 0.22 µm filter and kept at -20 °C until use.

4.2.4. Total phenolic content assay

Total phenolic content (TPC) assay was performed according to the conventional method (Singleton and Rossi, 1965) with minor modifications (Tian et al., 2019). Briefly, 0.1 mL of the phenolic extract in 7.9 mL of deionized water was reacted with 0.5 mL of Folin- Ciocalteu reagent for 5 min. Then, 1.5 mL of 20% Na_2CO_3 was immediately added to the mixture. After 2 hours, the absorbance at 765 nm was recorded with a VWR UV1600-PC spectrophotometer (Radnor, PA, USA). The final TPC value was calculated using gallic acid as an external standard and expressed as microgram gallic acid equivalence (GAE) per gram of the bran sample (mg GAE/g).

4.2.5. Analysis of phenolic acid compositions

Phenolic extracts from wheat brans were analyzed using UPLC-DAD-ESI-Q-TOF-MS/MS instrument from Waters Corporation (Milford, MA, USA). Flow rate, mobile phase gradients, and MS parameters were the same as reported in our previous study (Tian et al., 2021). Simple phenolic acids (4-hydroxybenzoic acid, vanillic acid, syringic acid, *para*-coumaric acid, *trans*-ferulic acid, sinapic acid) were identified and quantified according to external analytical standards. Due to lack of analytical standard, isomers of di-ferulic acids (DFA) were identified according to previous studies (Gong et al., 2017) and quantified using the external standard curve of *trans*-ferulic acid at the absorbance of 280 nm.

4.2.6. Statistical analysis

Data analysis was performed using PROC GLIMMIX in SAS version 9.4 (Cary, NC, USA). Year, location (loc), wheat variety, managements and their interactions were considered as fixed effects. Replicate (rep), rep(year), rep(loc), man*rep(year) and man*rep (loc) were considered as random effects. Relevant orthogonal contrasts were evaluated using the Turkey's test in SAS. Principal component analysis (PCA) was performed by Unscrambler 11 software (Oslo, Norway).

4.3. Results and discussions

4.3.1. Weather conditions in field experiments

The 2017-18 growing season was extremely dry, with growing season precipitation representing only ~50-60% of the long-term precipitation. It had a cold and dry winter and early spring, and a hot and dry late spring and early summer. These conditions delayed the onset of the reproductive

stage of the crop, and accelerated late-season crop phenology, decreasing grain yield. Due to extremely dry conditions, the variation in yield as affected by management, genotype and location was relatively narrow, with grain yield ranging from 3.2 Mg ha^{-1} for the genotype WB4458 in Hutchinson under FP; to 6.2 Mg ha^{-1} for the same genotype in Belleville under the IPP. Meanwhile, the 2018-19 growing season had optimum weather conditions, with precipitation averaging ~150% of normal which, combined with cooler temperatures, allowed for increased wheat grain yield. However, these conditions also allowed for increased disease development, in particular stripe and leaf rust, as well as Fusarium head blight. Consequently, higher yielding conditions coupled with high disease pressure lead to a wider range in grain yield than that measured in the 2017-18 season, with minimum grain yield of 2.3 Mg ha^{-1} for the genotype WB4458 under FP in Belleville, and a maximum of 9.6 Mg ha^{-1} for the genotype WB-Grainfield in Leoti under Yw.

4.3.2. Summary of ANOVA analysis

Total phenolic content (TPC) is the most widely used assay method for characterization of wheat phenolic extract(Tian, Chen, Zhang, et al., 2021). Both phenolic acids and other bio-active compounds can contribute to the TPC value. Therefore, TPC assay can be especially useful and provide unique information when full characterization by LC-MS is not possible. Based on UPLC-DAD-ESI-Q-TOF-MS/MS analysis, we identified and quantified phenolic acids including 4-hydroxybenzoic acid, vanillic acid, syringic acid, *para*-coumaric acid, *trans*-ferulic acid, sinapic acid, *cis*-ferulic acid, and isomers of di-ferulic acids (DFA), namely, 8-8 DFA, 8-5 DFA, 5-5 DFA, 8-O-4 DFA and 8-5’ benzofuran DFA. *Trans*-ferulic acid was the most abundant phenolic acid in wheat, and the concentration of *trans*-ferulic acid (ranging from 1681 to 2752

μg/g bran) was much higher than the other phenolic acids (e.g., concentrations only ranged between 20 to 200 μg/g bran). Analysis of variance (ANOVA) results are summarized in **Table 4-1**. Year × location × management and year × genotype × management interactions were significant for TPC. Year × location × genotype × management interaction was significant for the concentration of *trans*-ferulic acid and major other phenolic acids such as vanillic acid, sinapic acid, and 5-5 DFA. Since the concentration of *trans*-ferulic acid was much higher than all the other phenolic acids, the following discussions are focused on the effect on TPC and concentration of *trans*-ferulic acid.

4.3.3. Interaction effects on TPC

Year × genotype × management interaction is shown in **Figure 4-1**. Least square mean values of TPC ranged from 5602.53 μg GAE/g (WB4458 under YW treatment in 2018) to 6227.99 μg GAE/g (WB-Grainfield under FP treatment in 2018). In 2018, which was a very dry year, treatments with no fungicide (FP and EF) had the highest TPC, and the TPC of WB-Grainfield and WB4458 did not differ greatly among the two treatments. One fungicide application (EI) decreased TPC more in WB-Grainfield than in WB4458 compared to EF, though not significantly different ($p > 0.05$); however, treatments receiving two fungicide applications (IFP and YW) decreased TPC more for WB4458 than for WB-Grainfield. In 2019, there was more precipitation which was usually considered suitable for plant growth but can also lead to increased risk of fungal diseases. The TPC of WB4458 was consistently higher than that of WB-Grainfield, especially for management practices with no fungicide application. This may be attributed to the fact that WB4458 is relatively more susceptible to diseases than WB-Grainfield in the studied years (Onofre et al., n.d.). Phenolic compound is one kind of plant secondary

metabolites that protect plants against microbial infections (Wallace, 2004). Especially for wheat, McKeehen et al. (1999) found that *trans*-ferulic acid and *para*-coumaric acid can significantly ($p < 0.05$) reduce the growth of Fusarium species. While the genotype WB4458 is overall more susceptible to fungal diseases than WB-Grainfield, this susceptibility is even greater for Fusarium head blight, a disease experienced during the 2018-2019 growing season (Hollandbeck et al., 2019). Perhaps this susceptibility partially explains the greater accumulation of phenolic compounds as a response to growth environments more favorable to the pathogen. This can be supported by the fact that EF and FP managements with no fungicide application had the highest TPC than the groups with fungicide applications. To our knowledge, this the first study that reported enhanced accumulation of phenolic compounds in wheat under no fungicide application, though this effect was also dependent on other environmental factors and on wheat genotype. This finding may also partially explain previous reported observations that organically grown wheat contained higher concentration of phytochemicals than conventionally grown wheat (Mazzoncini et al., 2015; Zuchowski et al., 2011).

Year × location × management interaction is shown in **Figure 4-2**. In 2018, wheat grown in Belleville generally exhibited higher TPC values than in other two locations, especially for the FP management. But in 2019, wheat grown in Hutchinson exhibited higher TPC values while the difference among locations was most apparent for EF treatment. Compared with year × genotype × management interaction in **Figure 4-1**, higher differences in TPC were observed in the year × location × management plot **(Figure 4-2):** the TPC ranged from 4920.17 μg GAE/g (IFP at Belleville location in 2019) to 6797.37 μg GAE/g (FP at Belleville in 2018). It seemed that although both three-order interactions were significant, location variance had a stronger effect

than genotype variance in this study. Several previous studies also reported that environment effects were considerably larger than genotype effects (Bellato et al., 2013; Moore et al., 2006; Mpofu et al., 2006).

4.3.4. Interaction effect on *trans*-ferulic acid

Fourth-order interaction of year × location × genotype × management was significant for concentration of *trans*-ferulic acid (**Figure 4-3**). Letters denoting significance of difference from Turkey's test are presented in **Table 4-2**. The lowest value was observed for WB4458 in 2019 at Belleville location under IFP treatment (1721.90 µg/g bran), and the highest value was observed for WB4458 in 2019 at Hutchinson location under EF treatment (2774.54 µg/g bran). The year × location interaction is obvious in this plot: at Belleville, wheat from 2018 contained much higher *trans*-ferulic acid than corresponding wheat from 2019 for all the managements; however, at Hutchinson, the trend was reversed as wheat from 2019 contained higher concentration of *trans*-ferulic acid than that from 2018 for all management practices. At Leoti, the year interaction was not apparent but effect from wheat genotype was more apparent: WB-Grainfield contained higher concentrations of trans-ferulic acid than WB4458. We note that while the total amount of N available in the season was the same for a given treatment across locations (based on yield goals), the amount of inorganic N fertilizer added depended on location based on initial soil NO_3-N amount available. Interestingly, inorganic N rate was the lowest at Belleville in 2018 and at Hutchinson in 2019 (**Table 4-3**) because these locations had the highest original N level in the soil within their respective years. Though the total nitrogen rate was balanced according to the yield goal of each management system there was a strong positive correlation ($R^2 = 0.65$) between original N level and concentration of *trans*-ferulic acid (**Figure 4-4**). Future research

should focus on providing more empirical evidence and mechanistic explanation behind this relationship.

In section 4.3.3, we discussed that fungicide application reduced TPC of wheat. Since *trans*-ferulic acid was the most abundant phenolic compound in the bran extract and the major contributor to TPC, it was expected that fungicide application would negatively influence concentration of *trans*-ferulic acid. There were some observations supporting this expectation. For example, WB4458, which was more susceptible to fungi infection, exhibited a higher concentration of *trans*-ferulic acid in 2019 at Hutchinson for EF management (no fungicide application). Also, in 2018 at Belleville, both WB-Grainfield and WB4458 demonstrated relatively higher concentration of *trans*-ferulic acid in EF and FP management (with no fungicide application) than other treatments with fungicide applications. However, at Leoti, there was no clear relation between fungicide application and *trans*-ferulic acid concentration. We note that the experiment in Leoti was conducted amidst a commercial production field that received two fungicide applications, reducing the overall inoculum of fungal diseases in this location (see *Box 1* in Munaro et al., 2020). Additionally, it was possible that there were other bio-active compounds produced under fungi infection contributing to the increased TPC values besides *trans*-ferulic acid. Taken together, ANOVA suggested that the year × location × genotype × management was significant for concentration of *trans*-ferulic acid, but the interaction pattern was rather complicated. Further, other factors such as starting N level in the soil or other soil and environmental conditions could also played an important role in determining the phenolic acid concentration.

4.3.5. Effects on other phenolic acids

Similar to *trans*-ferulic acid, concentration of 4-hydroxybenzoic acid, vanillic acid, para-coumaric acid, sinapic acid, *cis*-ferulic acid were also significantly influenced by year × location × management × genotype interaction. Year × location × management × genotype interaction was not significant for concentration of 8-8 DFA and 8-O-4 DFA. Noteworthy, field management practices showed high impact on concentration of most phenolic acids and are presented in **Table 4-5**. Conventional farmer's practices (FP) significantly ($p < 0.05$) promoted accumulation of 4-hydroxybenzoic acid, para-coumaric acid, and cis-ferulic acid as compared to the other practices. Groups with no fungicide applications (farmer's practice or enhanced fertility) also contained higher concentrations of sinapic acid, 8-8 DFA, 8-5 DFA, 5-5 DFA, and 8-5' benzofuran DFA than treatments with fungicide application, though some of these differences are not significant.

Correlations were observed between concentration of individual phenolic acid (**Table 4-5**). For example, concentration of *trans*-ferulic acid and sinapic acid were positively correlated (r = 0.78); concentration of 8-8 DFA and 8-O-4 DFA were also positively correlated (r = 0.74); and concentration of *cis*-ferulic acid and of *para*-coumaric acid were negatively correlated (r = -0.59).

4.4. Conclusions

In this study, we investigated effect of wheat managements, wheat variety, environmental factors and their interactions on phenolic profile of wheat brans. Our results suggested that year x location x management and year x management x variety interactions were significant for total

phenolic content (TPC). Year x location x management x variety interaction was significant for concentration of *trans*-ferulic acid. Managements with no fungicide application (i.e. farmer' s practice and enhanced fertility) generally led to increased accumulation of phenolic compounds. Further, higher original nitrogen level in the soil may positively affect TPC and phenolic acids concentrations. More studies are needed to gain further understanding on effect of each treatment in the integrated managements and its interactions with other factors on phenolic profile of bran and wheat whole grains.

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Conflict of interest

The authors declare that there is no known conflict of interests.

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Figures and tables

Table 4-1 Treatment description of five management strategies which consisted of a farmer practice (FP) and the addition of four inputs (enhanced fertility (EF), economical intensification (EI), increased foliar protection (IFP), water-limited yield potential (Yw).

Treatments	FP	EF	EI	IFP	Yw
Nitrogen rate for yield goal	2.4 Mg ha-1	6.7 Mg ha-1	6.7 Mg ha-1	6.7 Mg ha-1	6.7 Mg ha-1
Seeding rate (million seeds ha^{-1})	2.7	2.7	2.7	2.7	2.7
In-furrow starter fertilizer	No	Yes	Yes	Yes	Yes
Fungicide growth stage application (Feekes scale)	No	No	10.5	6 and 10.5	6 and 10.5
Micronutrients	No	No	No	No	Yes

Table 4-2 Summary of analysis of variance (ANOVA) results.

Effect	TPC	TFA	4-HA	VA	PCA	SIPA	CFA	8-8 DFA	8-5 DFA	5-5 DFA	8-O-4 DFA	8-5' BDFA
year	ns	ns	***	*	**	ns	***	*	*	***	**	***
loc	*	***	***	ns	***	***	***	***	ns	ns	ns	**
var	**	ns	***	***	***	***	***	**	***	***	***	***
man	**	***	***	*	***	***	***	**	ns	*	***	ns
year x loc	***	***	***	***	***	***	***	***	***	***	***	***
year x man	*	***	***	**	***	**	***	ns	*	***	**	*
year x var	***	***	ns	***	***	***	***	ns	***	ns	ns	*
loc x man	***	***	***	***	***	***	***	***	***	***	***	*
loc x var	***	***	***	***	***	***	***	***	***	***	***	***
man x var	*	***	***	***	***	***	***	**	***	ns	ns	**
year x loc x man	***	***	***	***	***	***	***	**	***	**	***	**
year x loc x var	ns	***	***	*	***	***	***	**	ns	***	ns	ns
year x man x var	**	***	***	ns	***	***	***	ns	ns	ns	*	ns
loc x man x var	ns	***	***	***	***	***	***	***	**	ns	**	*
year x loc x man x var	ns	***	***	***	***	***	***	ns	*	***	ns	**

Loc=location; var=variety; man=management; *, ** and *** denote significance difference at confidence level of <0.05, < 0.01 and < 0.001 respectively; ns= not significant. TPC: total phenolic content; 4-hydroxybenzoic acid: 4-HA; vanillic acid: VA; syringic acid: SyrA; *para*-coumaric acid: PCA; *trans*-ferulic acid: TFA; sinapic acid (SIPA); *cis*-ferulic acid: CFA.

Table 4-3 Tukey-Kramer grouping for year*loc*man*gen least squares means (alpha=0.05).

year	loc	man	gen	Estimate										
2019	**Hutchins**	**EF**	**WB4458**	2774.54							A			
2019	**Hutchins**	**EI**	**Grainfie**	2580.95							B			
											B			
2019	**Hutchins**	**YW**	**WB4458**	2521.32			C				B			
							C				B			
2019	**Hutchins**	**EI**	**WB4458**	2515.00			C				B			
							C				B			
2019	**Hutchins**	**IFP**	**WB4458**	2511.06			C				B		D	
							C				B		D	
2019	**Hutchins**	**FP**	**WB4458**	2475.93			C		E		B		D	
							C		E				D	
2019	**Hutchins**	**YW**	**Grainfie**	2467.55			C		E		F		D	
							C		E		F		D	
2019	**Hutchins**	**EF**	**Grainfie**	2453.95			C		E		F		D	
							C		E		F		D	
2018	**Belllevi**	**FP**	**WB4458**	2440.72			C		E		F		D	
									E		F		D	
2018	**Belllevi**	**FP**	**Grainfie**	2404.65			G		E		F		D	
							G		E		F			
2019	**Hutchins**	**IFP**	**Grainfie**	2396.84			G		E		F		H	
							G		E		F		H	
2018	**Belllevi**	**EF**	**WB4458**	2369.30			G		E		F		H	
							G				F		H	
2018	**Belllevi**	**EI**	**WB4458**	2357.94			G		I		F		H	
							G		I				H	
2018	**Belllevi**	**IFP**	**WB4458**	2308.85			G		I		J		H	
									I		J		H	
2018	**Belllevi**	**IFP**	**Grainfie**	2292.89			K		I		J		H	
							K		I		J			
2019	**Leoti**	**YW**	**Grainfie**	2258.98			K		I		J		L	
							K		I		J		L	
2019	**Hutchins**	**FP**	**Grainfie**	2254.43			K		I		J		L	
							K		I		J		L	
2018	**Belllevi**	**YW**	**WB4458**	2254.03			K		I		J		L	
							K		I		J		L	
2018	**Leoti**	**EF**	**Grainfie**	2251.05			K		I		J		L	
							K		I		J		L	
2018	**Belllevi**	**EI**	**Grainfie**	2250.93			K		I		J		L	
							K				J		L	

year	loc	man	gen	Estimate										
2018	Leoti	YW	Grainfie	2246.22			K		M		J		L	
							K		M		J		L	
2018	Belllevi	EF	Grainfie	2224.66	N		K		M		J		L	
					N		K		M		J		L	
2018	Hutchins	YW	Grainfie	2224.56	N		K		M		J		L	
					N		K		M		J		L	
2019	Leoti	IFP	Grainfie	2221.98	N		K		M		J		L	O
					N		K		M		J		L	O
2018	Hutchins	IFP	Grainfie	2208.20	N		K		M		J	P	L	O
					N		K		M		J	P	L	O
2018	Leoti	IFP	Grainfie	2201.21	N		K		M	Q	J	P	L	O
					N		K		M	Q	J	P	L	O
2019	Leoti	YW	WB4458	2198.85	N		K		M	Q	J	P	L	O
					N		K		M	Q		P	L	O
2018	Hutchins	EF	Grainfie	2184.73	N		K		M	Q	R	P	L	O
					N				M	Q	R	P	L	O
2018	Hutchins	EI	Grainfie	2177.24	N		S		M	Q	R	P	L	O
					N		S		M	Q	R	P	L	O
2018	Leoti	FP	Grainfie	2169.36	N		S	T	M	Q	R	P	L	O
					N		S	T	M	Q	R	P	L	O
2018	Leoti	EI	Grainfie	2158.88	N	U	S	T	M	Q	R	P	L	O
					N	U	S	T	M	Q	R	P		O
2018	Hutchins	EI	WB4458	2139.41	N	U	S	T	M	Q	R	P	V	O
					N	U	S	T	M	Q	R	P	V	O
2019	Belllevi	YW	WB4458	2138.77	N	U	S	T	M	Q	R	P	V	O
					N	U	S	T		Q	R	P	V	O
2019	Leoti	EI	Grainfie	2129.71	N	U	S	T		Q	R	P	V	O
					N	U	S	T		Q	R	P	V	O
2018	Leoti	EF	WB4458	2122.73	N	U	S	T	W	Q	R	P	V	O
					N	U	S	T	W	Q	R	P	V	O
2019	Leoti	FP	Grainfie	2121.58	N	U	S	T	W	Q	R	P	V	O
					N	U	S	T	W	Q	R	P	V	O
2018	Hutchins	IFP	WB4458	2116.12	N	U	S	T	W	Q	R	P	V	O
						U	S	T	W	Q	R	P	V	O
2018	Hutchins	FP	WB4458	2114.02		U	S	T	W	Q	R	P	V	O
						U	S	T	W	Q	R	P	V	
2018	Hutchins	EF	WB4458	2105.07	X	U	S	T	W	Q	R	P	V	
					X	U	S	T	W	Q	R		V	
2018	Belllevi	YW	Grainfie	2094.79	X	U	S	T	W	Q	R		V	Y
					X	U	S	T	W		R		V	Y
2019	Belllevi	EF	WB4458	2078.56	X	U	S	T	W		R		V	Y
					X	U	S	T	W				V	Y
2018	Hutchins	FP	Grainfie	2072.57	X	U	S	T	W		Z		V	Y
					X	U		T	W		Z		V	Y
2019	Leoti	IFP	WB4458	2066.09	X	U		T	W		Z		V	Y

year	**loc**	**man**	**gen**	**Estimate**									
					X	U			W		Z		V	Y
2019	**Leoti**	**EF**	**Grainfie**	2058.43	X	U	A		W		Z		V	Y
					X	U	A		W		Z		V	Y
2019	**Leoti**	**EF**	**WB4458**	2054.26	X	U	A		W		Z		V	Y
					X	U	A		W		Z		V	Y
2019	**Belllevi**	**FP**	**Grainfie**	2053.76	X	U	A		W		Z		V	Y
					X		A		W		Z		V	Y
2019	**Leoti**	**FP**	**WB4458**	2045.80	X		A		W		Z		V	Y
					X		A		W		Z			Y
2019	**Belllevi**	**FP**	**WB4458**	2019.13	X		A		W		Z		B	Y
					X		A		W		Z		B	Y
2018	**Leoti**	**YW**	**WB4458**	2014.59	X		A		W		Z		B	Y
					X		A				Z		B	Y
2019	**Leoti**	**EI**	**WB4458**	2001.27	X		A				Z		B	Y
					X		A				Z		B	Y
2018	**Leoti**	**EI**	**WB4458**	1999.78	X		A				Z		B	Y
							A				Z		B	Y
2018	**Leoti**	**FP**	**WB4458**	1989.80			A				Z		B	Y
							A				Z		B	
2018	**Leoti**	**IFP**	**WB4458**	1968.36			A		C		Z		B	
							A		C				B	
2018	**Hutchins**	**YW**	**WB4458**	1955.19			A		C		D		B	
									C		D		B	
2019	**Belllevi**	**EI**	**Grainfie**	1934.12					C		D		B	
									C		D			
2019	**Belllevi**	**EF**	**Grainfie**	1867.06			E		C		D			
							E				D			
2019	**Belllevi**	**EI**	**WB4458**	1850.31			E				D		F	
							E						F	
2019	**Belllevi**	**YW**	**Grainfie**	1788.90			E				G		F	
											G		F	
2019	**Belllevi**	**IFP**	**Grainfie**	1741.81							G		F	
											G			
2019	**Belllevi**	**IFP**	**WB4458**	1721.90							G			

Table 4-4 Correlation between concentrations of different phenolic acids.

Variable Correlation	4-HA	VA	PCA	TFA	SIPA	CFA	8-8 DFA	8-5 DFA	5-5 DFA	8-O-4 DFA	8-5' BDFA
4-HA	1	-0.19	0.30	0.41	0.36	-0.15	0.50	0.51	0.14	0.39	0.25
VA	-0.19	1	0.44	-0.04	-0.09	-0.15	0.01	0.19	0.05	0.40	0.46
PCA	0.30	0.44	1	0.41	0.34	-0.59	0.35	0.50	0.38	0.53	0.24
TFA	0.41	-0.04	0.41	1	0.78	-0.15	0.53	0.66	0.35	0.28	0.02
SIPA	0.36	-0.09	0.34	0.78	1	-0.29	0.25	0.60	0.16	0.10	-0.05
CFA	-0.14	-0.15	-0.59	-0.15	-0.28	1	-0.29	-0.02	-0.05	-0.50	-0.02
8-8DFA	0.50	0.01	0.35	0.53	0.25	-0.29	1	0.33	0.34	0.74	0.25
8-5DFA	0.51	0.19	0.50	0.66	0.60	-0.02	0.33	1	0.21	0.37	0.53
5-5 DFA	0.14	0.05	0.38	0.35	0.16	-0.05	0.34	0.21	1	0.30	-0.17
8-O-4 DFA	0.39	0.40	0.53	0.28	0.10	-0.50	0.74	0.37	0.30	1	0.56
8-5' BDFA	0.25	0.46	0.24	0.02	-0.05	-0.02	0.25	0.53	-0.17	0.56	1

4-hydroxybenzoic acid: 4-HA; vanillic acid: VA; *para*-coumaric acid: PCA; *trans*-ferulic acid: TFA; sinapic acid (SIPA); *cis*-ferulic acid: CFA; DFA: diferulic acid; BDFA: benzofuran diferulic acid.

Table 4-5 Effect of management practices on concentration of phenolic acids.

	FP	EF	EI	IFP	YW
4-HA(µg/g)	**67.34a**	64.96b	65.52b	63.45c	65.49b
VA(µg/g)	109.51ab	110.82ab	112.30ab	114.59a	105.58b
SYRA(µg/g)	24.52b	27.56a	27.10a	26.41ab	26.32ab
PCA(µg/g)	**49.16a**	46.43c	47.31b	44.37d	44.79d
TFA(µg/g)	2180.15b	**2212.03a**	2174.63b	2164.28c	2180.31b
SIPA(µg/g)	117.15a	120.19a	119.02a	116.70a	112.54b
CFA(µg/g)	**105.88a**	94.36c	100.15b	93.67c	95.18c
8-8DFA(µg/g)	**21.67a**	21.33ab	20.82abc	20.07c	20.56bc
8-5DFA(µg/g)	**51.94a**	50.82a	51.61a	50.32a	50.93a
5-5DFA(µg/g)	**85.74a**	85.25ab	83.41ab	81.49b	83.91ab
8-O-4DFA(µg/g)	157.05ab	**158.90a**	154.53bc	153.15c	154.83bc
8-5'BDFA(µg/g)	**76.17a**	72.73a	74.70a	73.21a	73.12a

Within each row, numbers with no letters in common are significantly different ($p < 0.05$).

Farmer practice (FP), enhanced fertility (EF), economical intensification (EI), increased foliar protection (IFP), water-limited yield potential (Yw). 4-hydroxybenzoic acid: 4-HA; vanillic acid: VA; *para*-coumaric acid: PCA; *trans*-ferulic acid: TFA; sinapic acid (SIPA); *cis*-ferulic acid: CFA; DFA: diferulic acid; BDFA: benzofuran diferulic acid.

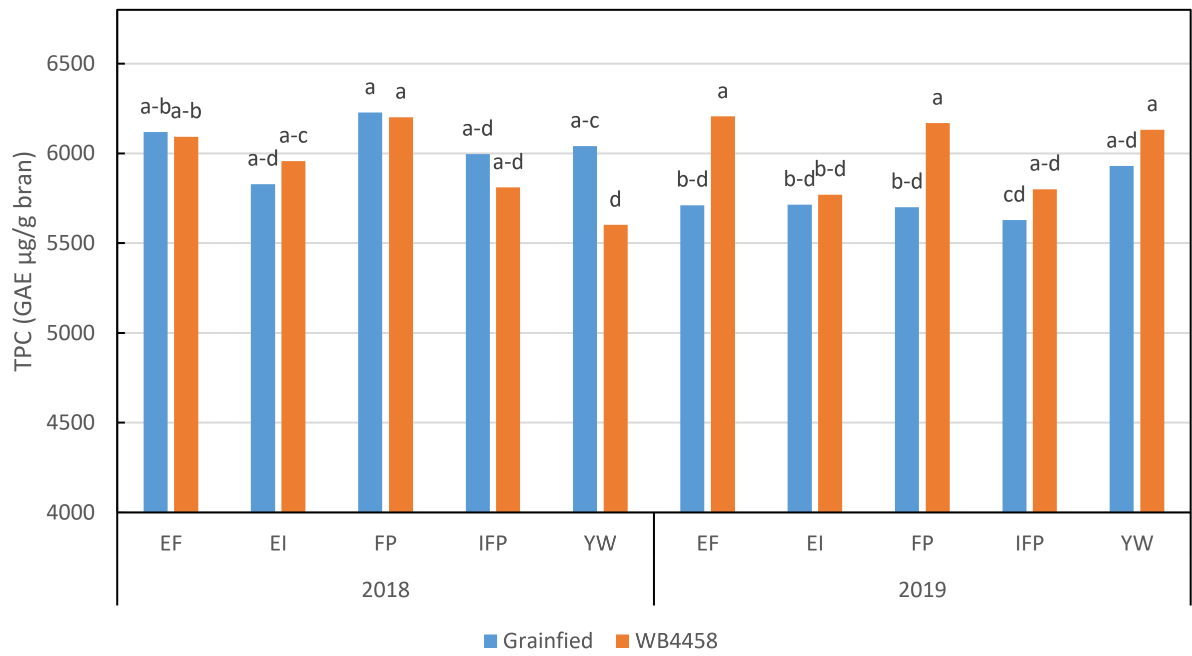


Figure 4-1 Effect of year x variety x management interaction on total phenolic content (TPC) of wheat brans.

Columns with no letter in common are considered as significantly different (P< 0.05). GAE: gallic acid equivalence.

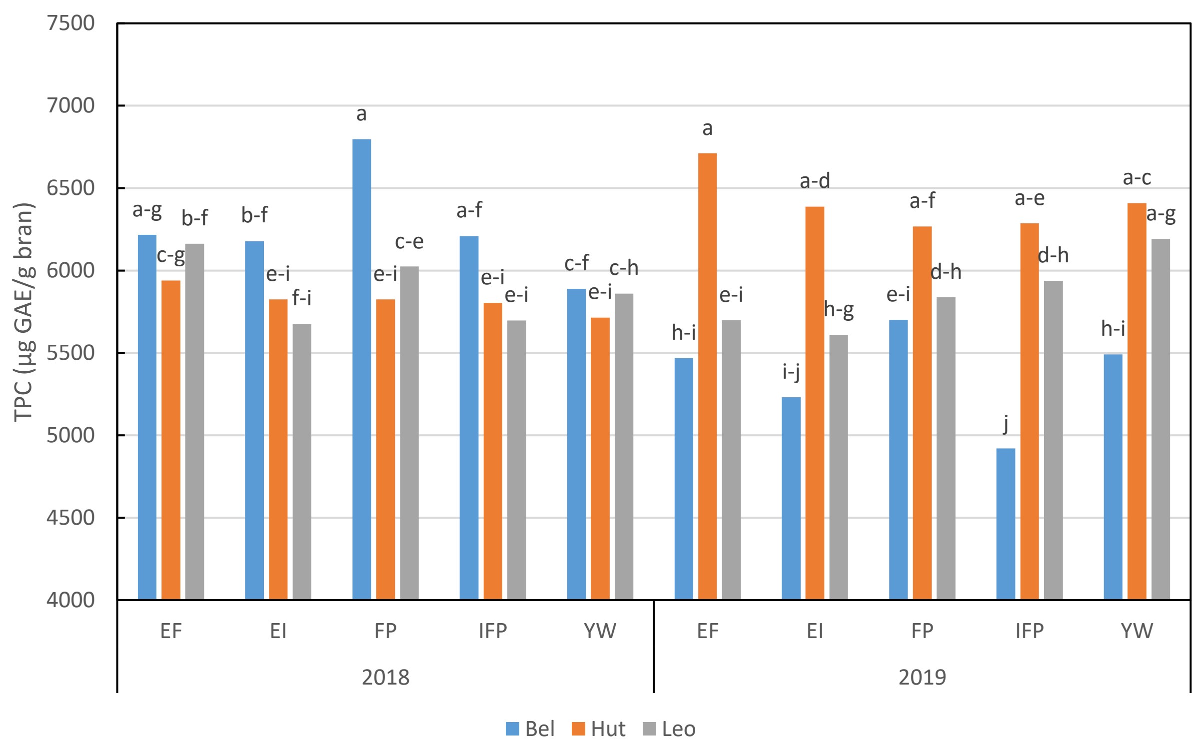


Figure 4-2 Effect of year x location x management interaction on TPC of wheat brans. Columns with no letter in common are considered as significantly different (P<0.05). GAE: gallic acid equivalence.

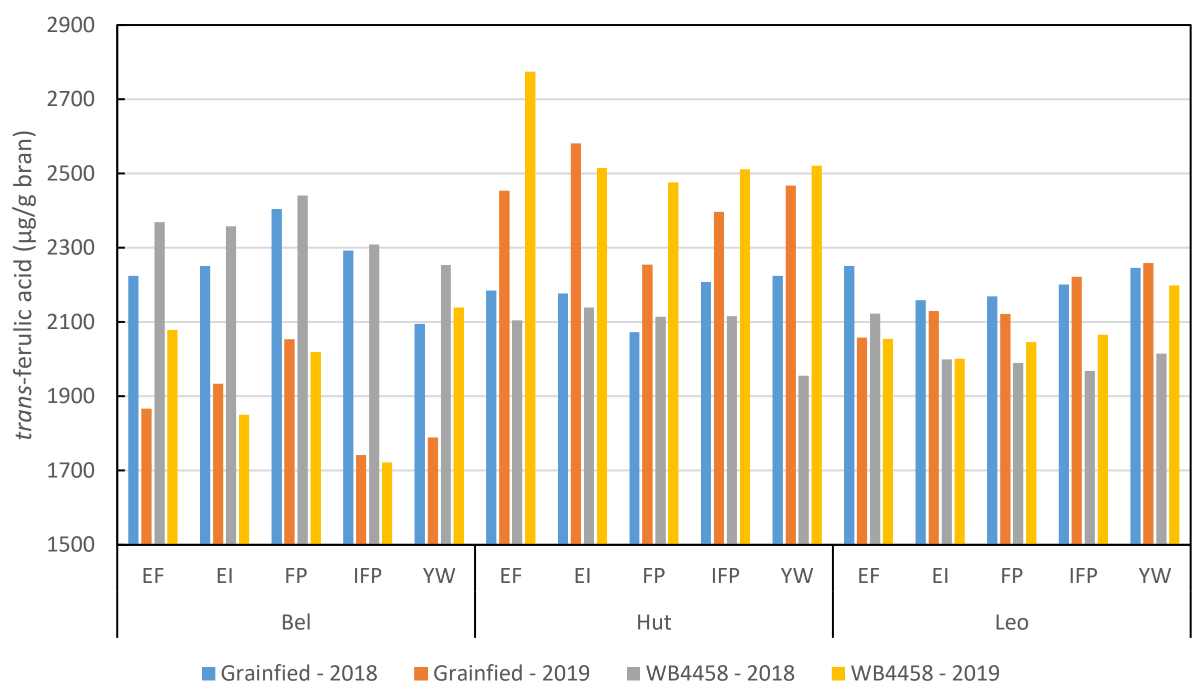
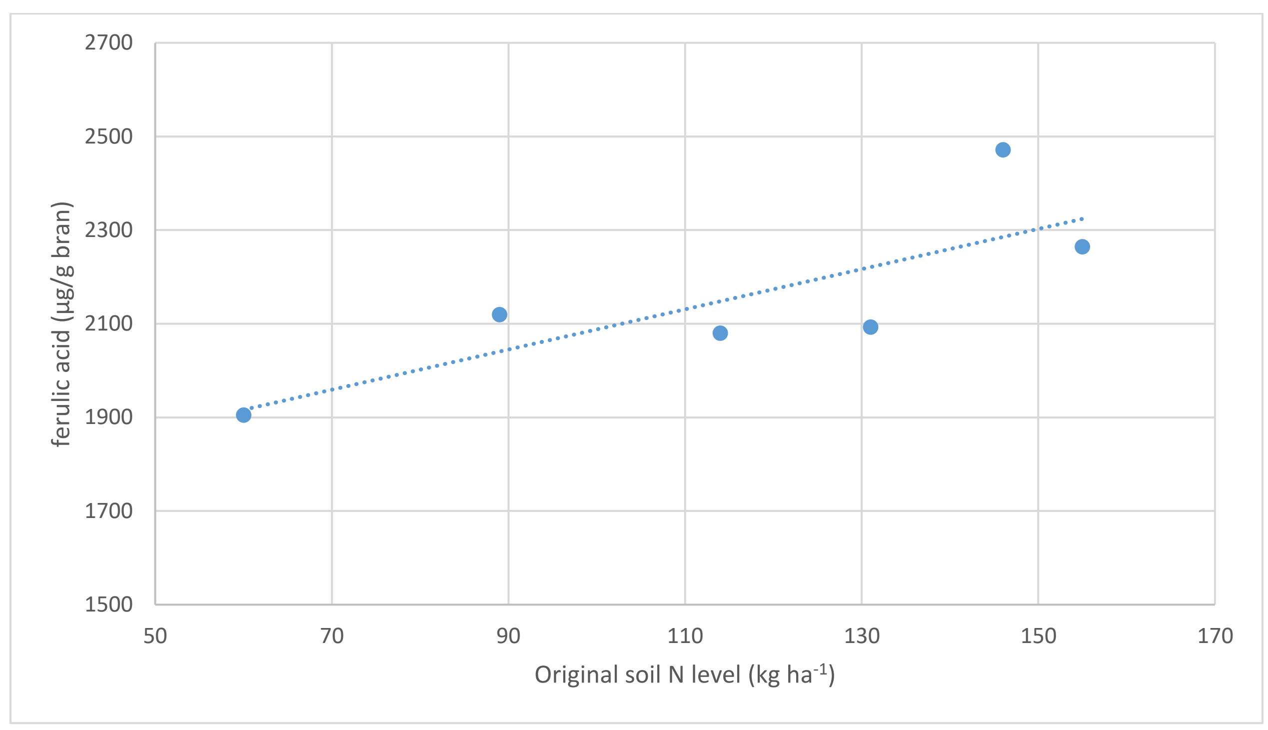


Figure 4-3 Year x location x variety x management interaction on concentration of *trans*-ferulic acid.

Figure 4-4 Correlation between original soil N level and concentration of trans-ferulic acid.



Chapter 5 - Changes in bread quality, antioxidant activity, and phenolic acid composition of wheats during early-stage germination*

Abstract

This study reported changes in baking properties, total phenolic content, antioxidant activity, and phenolic acid composition of three hard red winter wheat varieties during the early stage of seed germination. The wheats were sprouted at 30 °C and 95% relative humidity to achieve different germination levels based on falling number ranges (550 seconds for control flour; 350 (low), 250 (medium), and 120 (high) seconds for sprouted flours, respectively). Average germination times were 7, 8, and 10 h for the low, medium and high germinated samples, respectively. Most baking properties of sprouted whole flour were comparable to the control flour. However, total phenolic content, flavonoid content, phenolic acids, as well as antioxidant activity of sprouted flour were lower than the control flour. To our knowledge, this is the first study that reported both baking properties and antioxidant potential of sprouted whole wheat flour from early-stage germination. The study deepens the understanding of seed germination and the potential use of sprouted flour in baking industry.

Key Words: Early-stage germination; sprouted wheat; baking property; antioxidant activity; phenolic acid composition

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5.1. Introduction

Consumption of whole wheat products has been associated with reduced risks of chronic diseases (Gil et al., 2011; Tighe et al., 2010; Vitaglione et al., 2015). Health benefits of whole wheat are attributed to its various nutrients including vitamins, minerals, dietary fiber, and phytochemicals (Liu, 2007). Nutrient composition of whole wheat has been reported to be affected by the germination process (Aborus et al., 2018; Alvarez-Jubete et al., 2010). Simple phenolic acids are one group of important phytochemicals in wheat and are considered to be an important source of natural antioxidants (Hung, 2016). Possible antioxidative mechanisms include free radical scavenging, metal-chelating and glutathione peroxidase (Battin & Brumaghim, 2009).

Due to its potential health benefits, there have been increasing studies on either nutritional values or end-use properties of sprouted whole wheat flour. F. Yang (2001) found that the amount of ferulic acid and vanillic acid decreased slightly on day one of germination then significantly increased gradually to maximum levels on day seven of germination after 24 or 48 hours of steeping. Similarly, Žilić et al. (2014) found that total phenolic content (TPC), total radical scavenging activity and amount of ferulic acid, isoferulic acid, *p*-coumaric, and caffeic acid increased after 5 days of germination. Hung et al. (2011) tested two wheat varieties and reported changes of 4-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, and sinapic acid, as well as DPPH radical scavenging activity after 24 hours of steeping and two days of germination. They also found that TPC decreased after 2 days of germination for all the varieties while bound ferulic acid decreased in one variety and increased in other varieties.

Though it was generally agreed that baking properties of highly sprouted flour were impaired (McCleary & Sturgeon, 2002), sprouted wheat could find some advantages for baking purpose. It was reported that sprouted flour (72-90 hours of germination) added at a low percentage (1.5%) to un-sprouted flour enhanced dough properties (Marti et al., 2017). Ding et al. (2018) reported that whole wheat flour from 5-15 hours germinated wheat showed improved functional properties, i.e., less starch retrogradation and improved gluten quality and mixing tolerance. Other two studies reported that controlled-germinated flour improved loaf volume as well as crumb texture (Bellaio et al., 2013; Richter et al., 2014). Based on these results, baking properties of sprouted wheat flour seem to be influenced by many factors including wheat variety, sprouting methods and baking methods. Effect of sprouting on bread baking properties should be more thoroughly investigated.

Previous investigations of nutraceutical values of sprouted flour were focused mostly on germination for one to seven days, little information is available about the changes during the early stage germination, which is defined as germination time less than 12 h in this study. Meanwhile, metal-chelation is another important mechanism for antioxidant activity, but metal-chelation activity of sprouted wheat flour has not been reported. In addition, previous studies only considered either nutraceutical values or baking properties of sprouted flour. However, both aspects need to be considered for the production of functional foods using sprouted flour. In this study, phenolic acid composition, total phenolic content, flavonoid content, antioxidant activity and baking properties of sprouted and control whole wheat flour were determined. Our results will provide important information about changes in nutraceutical value and baking properties of

whole wheat during early-stage germination that has yet been available. These results will help deepen the understanding of germination process as well as evaluate the potential use of sprouted whole wheat flour in the production of functional foods.

5.2. Materials and methods

5.2.1. Chemicals and reagents

2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2′-bipyridyl, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), Disodium ethylenediaminetetraacetate (EDTA), fluorescein (FL), Trolox, and phenolic acid standards were purchased from Sigma-Aldrich (St. Louis, MO). Catechin standard, trifluoroacetic acid (TFA), water (HPLC grade), acetonitrile (I) (HPLC grade), and other general chemicals were purchased from Fisher Scientific (Hampton, NH).

5.2.2. Wheat samples and germination methods

Three hard red winter (HRW) wheat varieties Byrd, T158, and Tam 204 were obtained from Ehmke Seed (Healy, KS) from the 2017 crop year. Byrd has good bread baking characteristics. T158 has acceptable baking properties. Tam 204 was developed primarily for grazing and therefore has poor baking quality. To prepare each sprouted sample, 700 g wheat was first immersed into 1400 mL1.25% sodium hypochlorite for 30 minutes at room temperature and then rinsed with tap water for 30 minutes. The wheat was then rinsed twice with distilled water. 1400 mL water was added to the rinsed wheat for soaking. After the soaking phase, the wheat was spread evenly over the sprouting tray. The fermentation cabinet was set at 30°C and 95% relative humidity. Different varieties of wheat have different tolerances to germination. Therefore,

germination times for the different varieties were adjusted slightly to achieve desired falling numbers. On average, germination times were 7, 8, and 10 h, for the low, medium, and highly germinated samples, respectively. Level of germination was determined using the falling number (FN) test. The FN range for each degree of germination was 350 ± 40 s for low germination (L), 250 ± 40 s for medium germination (M) and 120 ± 40 s for high germination (H). The control, labeled as C, was sound wheat with an average falling number of 550 ± 40 s.

5.2.3. Whole wheat bread baking and analysis

Whole wheat bread was prepared using a modified method of AACCI Approved Method 10-10.03 (*AACC International Approved Methods - AACC Method 10-10.03. Optimized Straight-Dough Bread-Making Method*, n.d.). Bread was baked in replicate for each sample. Bread volume was measured immediately after baking using rapeseed displacement according to AACCI Approved Method10-05.01(*AACC International Approved Methods - AACC Method 10-05.01. Guidelines for Measurement of Volume by Rapeseed Displacement*, n.d.). A C-cell imaging system (Calibre Control International Ltd., Appleton, Warrington, United Kingdom) was used for the quantification of number of cells and cell wall thickness of the crumb. Crumb firmness and resilience were evaluated using the TA. XT Plus Texture Analyzer (Texture Technologies Corp., Scarsdale, NY, USA) according to AACCI Approved Method 74-10.02 (*AACC International Approved Methods -AACC Method 74-10.02. Measurement of Bread Firmness -- Compression Test*).

5.2.4. Extraction of soluble phenolic compounds

For each sample, 2 g whole grain flour was accurately measured and extracted with 25 mL 50% acetone for 20 hours under nitrogen protection and dark condition (Moore et al., 2005). The mixture was centrifuged at 4000 x*g* for 30 min. Supernatant was kept at room temperature under nitrogen protection and analyzed within 2 days.

5.2.5. Total phenolic content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu reagent (Xu et al., 2010). Briefly, 0.1 mL sample extract was mixed with 7.9 mL water and 0.5 mL 2 N Folin-Ciocalteu reagent for 5 minutes. Then 1.5 mL 20% sodium carbonate solution was added. The final mixture was left for 2 hours for color development at room temperature. The absorbance was measured at 765 nm. TPC was quantified using a gallic acid standard.

5.2.6. Total flavonoid content

Total flavonoid content (TFC) was determined following a previous method (Chlopicka et al., 2012), where 0.1 mL sample extract, 0.15 mL 5% $NaNO_2$ solution, and 2.4 mL water were mixed for 5 minutes. Then 0.15 mL $AlCl_3$ solution was added. After another 1 minute, 0.5 mL 2 M NaOH solution and 1.7 mL water were added. The absorbance was measured immediately at 510 nm. The final TFC was determined using a catechin standard curve.

5.2.7. DPPH assay

DPPH radical scavenging activity was measured using a method reported previously with some modifications (Pownall et al., 2010). A 0.5 mL aliquot sample extract was mixed with 5 mL100

μM DPPH solution. After 25 minutes, the mixture was centrifuged at 4000 xg for 5 minutes. The absorbance was measured at 517 nm and quantified using vitamin C as a standard.

5.2.8. ABTS assay

ABTS radical scavenging activity was determined using a previously reported method (Kong & Xiong, 2006). The final mixture contained 0.1 mL sample extract and 3.8 mL 0.13 mM ABTS radicals. The mixture was kept under dark condition for 10 minutes and the absorbance at 734 nm was recorded. Quantification was performed using Trolox as a standard.

5.2.9. Oxygen radical absorbance capacity (ORAC) assay

ORAC assay was performed using fluorescein as the fluorescent probe according to the method reported previously (Huang et al., 2002) using Biotek Synergy H1 Hybrid microplate reader (Winooski, VT, USA). Excitation and emission wavelengths were 485 and 528 nm, respectively. All reagents were prepared in a 75 mM phosphate buffer (pH 7.4) except for Trolox standards (prepared in deionized water). Sample extract was diluted 40 times before use. Fifty μL diluted sample was mixed with 100 μL 6nM fluorescein (FL) solution. The mixture was incubated at 37 °C for 30 minutes. Then 50 μL 76.5 mM AAPH solution was added. The fluorescence of the final mixture was recorded every minute for 2 h at 37 °C. Trolox equivalence (TE) were calculated using the relative area under the curve for samples compared to a Trolox standard curve prepared under the same experimental conditions.

5.2.10. Metal chelating assay

Metal chelating activity of sprouted flour was measured using a 2,2′-bipyridyl binding competition method (Yu et al., 2002). The final solution contained a mixture of 12.5 µL 1 mM $FeSO_4$ solution, 12.5 µL sample extracts, 50 µL 1M Tris-HCl buffer (pH 7.4), 50 µL 2,2'-bipyridyl solution (0.1% in 0.2 M HCl), 20 µL 10% hydroxylamine-HCl, and 100 µL ethanol. The mixture was allowed 30 minutes for color development. The absorbance was measured at 522nm using the Biotek Synergy H1 Hybrid Reader (Winooski, VT, USA) and quantified according to an external EDTA standard curve.

5.2.11. HPLC sample preparations and phenolic acid analysis

Soluble-free phenolic acid was extracted using a method reported previously (Moore et al., 2005) with some modifications in our lab (Tian & Li, 2018). Ten g flour was accurately measured and extracted twice with 30 mL acetone: methanol: water (7:7:6, v/v/v) each time. The mixture was centrifuged at 4000 x*g* for 10 minutes. The supernatants were combined and evaporated to a volume of 10 mL using a rotary evaporator. Five mL of the solution was kept for soluble-conjugate sample preparation. Another 5mL of the solution was extracted five times with ethyl acetate. The organic phase was combined and evaporated to dryness. The final residue was re-dissolved in HPLC grade methanol and kept at -70 °C until use.

Soluble-conjugate phenolic acid was prepared by NaOH hydrolysis of the soluble-free extract. Five mL soluble-free extract was hydrolyzed with 2 M NaOH for 3 hours under nitrogen and dark condition. The mixture was then acidified to pH=2 with 6 M HCl and extracted five times

with ethyl acetate. The combined organic phase was evaporated to dryness and then re-dissolved with HPLC grade methanol.

Insoluble-bound phenolic acid was obtained according to a previous report (Okarter et al., 2010). Two g flour was first extracted twice with acetone: methanol: water (7:7:6, v/v/v). After centrifugation, the solid residue was digested with 20 mL 2 M NaOH for 3 hours under nitrogen and dark condition. The mixture was centrifuged at 4000 xg for 10 minutes. Then the supernatant was acidified to pH=2 and extracted five times with ethyl acetate. The combined organic extract was evaporated to dryness and re-constituted with HPLC grade methanol.

Samples were analyzed using a reversed-phased HPLC system (Shimadzu, Kyoto, Japan) equipped with a Phenomenex F5 column (150mm x 4.6mm). Mobile phase A was 0.1% TFA in water and mobile phase B was 0.1% TFA in acetonitrile. The gradient elution was performed using a method reported previously with some modifications (Hung et al., 2011). The photo diode array (PDA) detector was set to scan signal from 220 to 800 nm. Resolved peaks were identified based on retention time and UV spectra compared with the standards. Quantifications of each phenolic acid was based on peak areas of external standards.

5.2.12. Statistical analysis

One-way analysis of variance (ANOVA) and Tukey’s tests were conducted for statistical analysis. Multivariate analysis of variance (MANOVA) test was also employed to analyze the effect of sprouting level, wheat genotypes as well as their interactions. $P < 0.05$ was considered

as significantly different. All calculations were performed using SAS institute's SAS software, version 9.3 (Cary, NC, USA).

5.3. Results and discussions

5.3.1. Bread-baking properties

Loaf volume and crumb properties of whole wheat bread are summarized in **Table 5-1**. Larger loaf volume is generally considered as superior baking quality for pan bread. For all three wheat varieties, there were no significant differences in loaf volume between the control group and sprouted groups. Number of cells, cell wall thickness, firmness and elasticity of bread crumb were measured for the evaluation of crumb quality. For T158, the highly germinated samples had significantly lower ($p<0.05$) number of cells than the other groups. For Byrd and Tam 204, there was no significant difference among the number of cells within each variety regardless of level of germination. For all three varieties, the cell wall thickness and crumb firmness were not significantly different between germination levels for each variety. However, for all the three varieties, the highly germinated sample was significantly less elastic than the corresponding control sample. Generally, these results suggested that early stage of seed germination did not impair bread-baking quality of the sprouted whole flour. Another recent study also reported that bread baked from control and 24-hour germinated flour exhibited similar loaf volumes (Baranzelli et al., 2018).

5.3.2. Total phenolic content

The total phenolic content (TPC) of tested whole wheat flour was expressed as mg gallic acid equivalence (GAE) per gram of flour (**Figure 5-1**). For Byrd, TPCs of the control group and low germination group were significantly greater than that of medium and high germinated groups ($p < 0.05$). For T158, the TPC of control group was significantly greater than the low and medium germinated groups ($p < 0.05$). For Tam 204, TPCs of different sprouting levels were similar. Our result also suggested that although germination level influenced TPC values, wheat variety is still the key factor determining the TPC. This was in agreement with previous studies comparing various wheat varieties (Loreto et al., 2018; Moore et al., 2005; Okarter et al., 2010).

Alvarez-Jubete et al. (2010) reported that TPC of control group wheat seed was 0.53mg GAE/ g. Lachman et al. (2011) reported that TPC of seven wheat varieties ranged from 0.522 to 0.787 GAE/ g. Yilmaz et al. (2015) reported that TPC of 15 wheat varieties ranged from 0.65 to 1.31mg GAE/ g. In our study, TPC of Byrd, T158 and Tam204 control group were 1.50, 1.87, 2.00 mg GAE/ g, respectively. Our reported values were higher than these studies. This might be due to different extraction protocols. Previous studies had found that many factors such as solvent used for extraction, extraction time, extraction temperature will influence the result of TPC measurement (Singh et al., 2012; Zhou & Yu, 2004).

5.3.3. Total flavonoid content

Besides simple phenolic acids, flavonoid compound is another important kind of phenolic compounds in wheat. For all the three varieties, the control group had significantly greater TFC values than the sprouted groups ($p < 0.05$) (**Figure 5-2**). This suggested that compared with other

phenolic compounds, flavonoid compounds were much less tolerant to the germination process. For Byrd, TFC decreased with increased level of germination. However, for T158 and Tam 204, TFC values of the different sprouted groups were very similar.

5.3.4. Radical scavenging activities

ABTS, DPPH, and ORAC assays were employed to study the radical scavenging activity of flour extracts (**Table 5-2**). Each assay has some limitations. ABTS and DPPH assays have been widely used; however, both assays only consider reaction stoichiometry but not antioxidant reaction rate and kinetic pattern. ORAC assay measures the kinetic pattern of the reaction but the complexity of the reaction mechanism could cause difficulties when performing this assay (Schaich et al., 2015). Therefore, it is advisable and necessary to use multiple assays for a comprehensive evaluation of antioxidant potential. Results of DPPH assay were expressed as μM vitamin C equivalence (VCE) per gram flour. Results of ABTS assay and ORAC assay were expressed as μM Trolox equivalence (TE) per gram flour.

In our study, ABTS values for Byrd, T158 and Tam 204 control group were 30.05, 28.13 and 28.13 μM TE/g sample, respectively. Moore et al. (2005) reported ABTS results of 8 soft wheat varieties ranged from 14.3 to 17.6 μM TE/g sample. Yilmaz et al. (2015) reported that ABTS values of 15 wheat varieties ranged from 9.4 to 14.3 μM TE/g sample. Results in this study were higher than previous studies. ORAC value for Byrd, T158 and Tam 204 control group was 41.57, 40.83 and 42.33 μM TE/g sample, respectively. Kim & Kim (2016) reported that ORAC values of wheat samples ranged from 54.8–71.8 μM TE/g. (Moore et al., 2005) reported that ORAC values of 8 soft wheat varieties were in the range of 32.9–47.7 μM TE/g. Our results were

comparable to the reports by (Moore et al., 2005) but lower than reported values by (Kim & Kim, 2016).

For the Byrd variety, radical scavenging activities of the control group were much greater those of sprouted groups using all three assays. Only slight differences of radical scavenging activity were noticed among values of the low, medium and highly germinated groups. For T158 variety, DPPH and ABTS values of the control group were much greater than those of sprouted groups ($p < 0.05$), and values of different sprouted levels were similar. However, no significant difference was observed for ORAC values. For Tam 204 variety, ABTS and ORAC values of control group were significantly greater ($p<0.05$) than those of sprouted groups. For DPPH activity, value of the low germination group was similar to control group. The medium and high germinated groups had significantly lower values than control group ($p < 0.05$).

In general, our results showed that radical scavenging activities of wheats decreased during the early stage of germination. Multiple assays showed similar pattern of change, but some differences existed. Highest percentage of reduction was observed for all varieties in ABTS radical scavenging activity. This observation showed that ABTS assay might be the most sensitive method to evaluate radical scavenging activities of sprouted wheat. ORAC values of T158 variety had the highest tolerance to germination. Interestingly, for all three varieties, radical scavenging activities from multiple assays of the medium and high germinated groups were always close. MANOVA analysis suggested that germination level, wheat variety as well as their interactions had significant effect on antioxidant activity results ($p <0.01$).

5.3.5. Metal chelating activity

Metal chelating is another mechanism responsible for the antioxidant activity. However, the metal chelating activity has not been reported in recent studies of sprouted wheat (Hung et al., 2011; Žilić et al., 2014). Metal chelating property was also reported not to be correlated with radical scavenging activities (Moore et al., 2005). Thus, change of metal-chelation property during the whole germination stage remains unknown. Results of the metal chelating activity of tested wheat samples are shown in **Figure 5-3**, expressed as milligram EDTA equivalence per gram flour. For Byrd variety, control group as well as low and medium germinated groups had significantly greater values than high germinated group ($p < 0.05$). For T158 variety, control group and medium germinated group had significantly greater values than high germinated groups ($p < 0.05$). For Tam 204 variety, control group had significantly greater values than medium and high germinated groups ($p < 0.05$). For all the three varieties, control groups always had significantly greater values than high germinated groups ($p < 0.05$).

5.3.6. Phenolic acid composition

Phenolic acids in cereal grains are distributed as soluble-free, soluble-conjugate, and insoluble-bound fractions (Adom et al., 2003). In this study, 4-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, and sinapic acid were detected in the extracts (**Table 5-3**).

Ferulic acid is the major phenolic acid in wheat flour. Insoluble-bound fraction accounted for more than 90% of the total ferulic acid. Brandolini et al. (2013) reported that amount of insoluble-bound ferulic acid was in the ranged of 420 to 610 μg/g sample. Okarter et al. (2010) reported that insoluble-bound ferulic acid of six wheat varieties ranged from 290 to 482 μg/g

sample. In our study, values of bound ferulic acid for Byrd, T158 and Tam 204 control group were 284, 294 and 291 μg/g, respectively.

For all the three varieties, ferulic acid in all the three fractions decreased significantly even with low level of germination. But the process was also dependent on wheat varieties. For Byrd, high germination level had lowest amount of ferulic acid but for Tam 204, ferulic acid content was greater in the highly germinated group than in the low and medium germinated groups. Previous studies also showed some controversy regarding changes of ferulic acid during the germination process. Hung et al. (2011) found that insoluble-bound ferulic acid increased in Canadian western red spring wheat but decreased in Canadian western Durum wheat. Žilić et al. (2014) found increased amount of ferulic acid after 5 days of germination. These results suggested that amount of ferulic acid decreased first and then increased to the maximum value after 7 days of germination. But pattern of change for the first few days was also influenced by different steeping times. In summary, change of ferulic acid was highly dependent on wheat varieties, steeping times and germination conditions.

Sinapic acid was found to be the major phenolic acid in the soluble-conjugate fraction (**Table 5-3**). Interestingly, amount of sinapic acid was not reported in many studies (Irmak et al., 2008; Mazzoncini et al., 2015; Okarter et al., 2010). The possible reason was that sinapic acid had very similar polarity and adsorption spectrum as ferulic acid, and it was not distinguished from ferulic acid in some studies. For Byrd and T158, amount of sinapic acid decreased significantly during the early stage of wheat germination. Tam 204 variety seemed to have relatively greater initial tolerance to the germination.

P-coumaric acid was another derivative of hydroxycinnamic acid found in wheat flour. The majority of *p*-coumaric acid was found in insoluble-bound fraction. For Byrd, total amount of *p*-coumaric acid started to decrease when germination reached medium level. For T158 and Tam 204, the amount of insoluble-bound *p*-coumaric did not change apparently with the germination process. Interesting, for soluble-conjugate fraction, *p*-coumaric acid decreased during early stage of germination for all the three varieties.

4-hydroxybenzoic acid, vanillic acid, and syringic acid were derivatives of hydroxybenzoic acid. Total amount of 4-hydroxybenzoic acid, vanillic acid and syringic acid were also quantified. Compared with 4-hydroxybenzoic acid and vanillic acid, greater percentage of reduction in total amount of syringic acid was observed during the early-stage germination process.

These results suggested that total amount of each phenolic acid decreased during the early stage of wheat germination, although vanillic acid and 4-hydroxybenzoic seemed to have relatively greater tolerance to the germination. For each phenolic acid, soluble-free, soluble-conjuagate and insoluble-bound fractions generally had similar patterns of change. This might suggest that during early stage of seed germination, changes of phenolic acid composition resulted from other metabolism activities instead of transformation among soluble-free, soluble-conjugate and insoluble-bound fractions.

The changes of individual phenolic acids were not in good correspondence with changes of TPC. This might be because that TPC assay was not specific to phenolic compounds. Other substances

in the extraction such as proteins and sugars could interfere the measurement (Margraf et al., 2015). Also, simple phenolic acid was only part of the phenolic compounds in wheat and there might be other phenolic compounds that contributed significantly to the TPC of the sample. No correlation has been found between phenolic acid composition and total phenolic content.

5.4. Conclusions

During the early stage of wheat germination, total phenolic content, total flavonoid content, and antioxidant activity decreased. Total amount of major phenolic acids also decreased. Sprouted flour showed comparable baking properties to sound flour. Controlled sprouting could change baking properties and nutraceutical values of whole wheat flour. However, further studies are necessary for the production of whole wheat flour with both improved end-use properties and increased health benefits.

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Conflict of interest

The authors declared no conflict of interest.

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Figures and tables

Table 5-1 Bread-baking properties of whole wheat flour samples.

Sample	Bread Volume(cc)	Crumb-Number of Cells	Crumb-Cell Wall Thickness(mm)	Crumb-Firmness(g)	Crumb-Elasticity (%)
Byrd C	756.0 ± 15.87a	3492 ± 70ab	0.433 ± 0.005cd	268 ± 17.34e	61.6 ± 0.79 a
Byrd L	777.0 ± 32.24a	3588 ± 196a	0.425 ± 0.011d	282 ± 30.76de	58.9 ± 1.55abcd
Byrd M	767.7 ± 44.22a	3567 ± 172a	0.431 ± 0.007cd	266 ± 6.85e	59.1 ± 0.55abc
Byrd H	764.3 ± 8.04a	3351 ± 70abc	0.443 ± 0.005abcd	294 ± 19.40cde	56.8 ± 0.66bcd
T158 C	702.7 ± 22.22ab	3071 ± 33cde	0.447 ± 0.005abc	311 ± 15.39bcde	60.1 ± 0.54ab
T158 L	695.3 ± 4.33abc	3169 ± 157bcd	0.439 ± 0.007cd	311 ± 22.69bcde	58.6 ± 1.46abcd
T158 M	696.7 ± 40.10abc	3108 ± 94cde	0.441 ± 0.003abcd	318 ± 35.04bcde	55.8 ± 1.65cde
T158 H	660.3 ± 50.19bc	2673 ± 174fg	0.459 ± 0.008a	313 ± 22.38bcde	52.6 ± 0.60ef
Tam 204 C	627.7 ± 45.62bc	2752 ± 128efg	0.446 ± 0.003abc	416 ± 62.26a	57.0 ± 2.03bcd
Tam 204 L	657.7 ± 15.21bc	2937 ± 176def	0.437 ± 0.009bcd	355 ± 11.98abcd	57.9 ± 0.97bcd
Tam 204 M	658.0 ± 21.79bc	2796 ± 24.56defg	0.447 ± 0.003abc	371 ± 11.29abc	55.6 ± 0.97de
Tam 204 H	610.3 ± 12.99c	2548 ± 88g	0.455 ± 0.007ab	387 ± 20.58ab	52.1 ± 0.87f

Results are expressed as mean ± SD, n= 2. For each property across all varieties, values with different letters are significantly different ($p<0.05$). C-control group (falling number 550±40 seconds), L-low sprouting level (falling number 350±40 seconds), M-medium sprouting level (falling number 250±40 seconds), H-high sprouting level (falling numbers 120±40 seconds).

Table 5-2 Radical scavenging activities of whole wheat flour samples.

Sample	ABTS (μM TE/g)	DPPH (μM VCE/g)	ORAC (μM TE/g)
Byrd C	30.047 ± 0.667a	6.041 ± 0.031b	41.573 ± 0.683ab
Byrd L	23.423 ± 0.127c	5.738 ± 0.038g	36.009 ± 0.487def
Byrd M	22.536 ± 0.660cd	5.955 ± 0.044ef	33.318 ± 1.041fg
Byrd H	22.841 ± 0.458cd	5.906 ± 0.037efg	34.002 ± 0.575fg
T158 C	28.134 ± 0.292b	6.426 ± 0.055b	40.832 ± 0.658abc
T158 L	21.843 ± 0.458de	6.176 ± 0.068cd	38.817 ± 1.811bcd
T158 M	21.289 ± 0.381e	5.894 ± 0.032efg	39.124 ± 0.651bc
T158 H	19.294 ± 0.249f	5.820 ± 0.154fg	38.352 ± 0.389cde
Tam204 C	28.134 ± 0.501b	7.269 ± 0.044a	42.333 ± 1.009a
Tam204 L	21.732 ± 0.790de	7.089 ± 0.046a	35.810 ± 0.582ef
Tam204 M	21.899 ± 0.566de	6.254 ± 0.031bc	32.253 ± 0.763g
Tam204 H	22.508 ± 0.315cde	6.053 ± 0.068de	32.775 ± 1.632g

Results are expressed as mean ± SD, n= 3. For each property across all varieties, values with different letters are significantly different ($p<0.05$). C-control group (falling number 550±40 seconds), L-low sprouting level (falling number 350±40 seconds), M-medium sprouting level (falling number 250±40 seconds), H-high sprouting level (falling numbers 120±40 seconds).

Table 5-3 Phenolic acid composition of whole wheat flour samples.

	Soluble-free (μg/g)	Soluble-conjugated (μg/g)	Insoluble- bound (μg/g)	Total soluble (μg/g)	Total (μg/g)
Ferulic acid					
Byrd C	2.116±0.287ab	11.545±0.044c	270.871±5.250a	13.661±0.287c	284.532±5.280a
Byrd L	1.161 ±0.071e	9.720± 0.049e	219.386±9.745c	10.881±0.117f	230.267±9.645c
Byrd M	1.293 ±0.115e	8.692± 0.176gf	196.021±2.475de	9.985±0.250gh	206.006±2.692de
Byrd H	1.239±0.095e	7.747± 0.439h	184.441±2.151e	8.986±0.519i	193.427±2.515e
T158 C	2.263±0.023a	13.264±0.410a	279.112±1.573a	15.527±0.387a	294.639±1.406a
T158 L	1.670±0.136cd	11.015±0.153cd	228.163±2.005bc	12.685±0.107d	240.848±1.955bc
T158 M	1.871±0.019bc	12.613±0.058b	244.167±1.046b	14.484±0.045b	258.651±1.088b
T158 H	1.400±0.102de	10.894±0.194d	216.105±0.953c	12.294±0.246de	228.399±0.880c
Tam204 C	2.158±0.154ab	9.514±0.065e	279.679±2.280a	11.672±0.096e	291.351±2.287a
Tam204 L	1.314±0.036de	8.163±0.060gh	224.560±0.594c	9.477±0.063gh	234.037±0.607c
Tam204 M	1.189±0.064e	8.215±0.042gh	212.037±0.890d	9.404±0.106hi	221.441±0.995cd
Tam204 H	1.219±0.107e	8.906±0.125f	245.258±0.489b	10.126±0.191g	255.384±0.680b
p-Coumaric acid					
Byrd C	ND	3.011±0.507a	15.861±0.001cd	3.011±0.507a	18.872±0.505cd
Byrd L	ND	1.643±0.014bc	19.010±1.821bc	1.643±0.014bc	20.654±1.835bc
Byrd M	ND	1.261±0.131bc	9.137±0.763e	1.261±0.131bc	10.398±0.632e
Byrd H	ND	1.069±0.148c	7.892±0.076e	1.069±0.148c	8.961±0.224e
T158 C	ND	3.047±0.028a	20.317±1.904bc	3.047±0.028a	22.759±1.876abc
T158 L	ND	1.519±0.116bc	27.982±3.096a	1.519±0.116bc	29.501±1.114a
T158 M	ND	1.581±0.028bc	20.828±3.004bc	1.581±0.028bc	22.409±3.032bc

T158 H	ND	1.247±0.003bc	12.256±0.889de	1.247±0.003bc	13.504±0.886de
Tam204 C	ND	1.943±0.223b	19.745±0.014bc	1.943±0.223b	21.688±0.236bc
Tam204 L	ND	1.006±0.045c	28.179±1.223a	1.006±0.045c	29.185±1.178a
Tam204 M	ND	1.084±0.0967c	17.098±0.055cd	1.084±0.0967c	18.182±0.041cd
Tam204 H	ND	1.166±0.277c	24.535±1.289ab	1.166±0.277c	25.702±1.012ab
Sinapic acid					
Byrd C	ND	30.231±0.035b	ND	30.231±0.035b	30.231±0.035ef
Byrd L	ND	20.927±3.152d	ND	20.927±3.152d	20.927±3.152fg
Byrd M	ND	16.375±1.447ef	ND	16.375±1.447ef	16.375±1.447g
Byrd H	ND	13.067±1.740f	ND	13.067± 1.740f	13.067±1.740g
T158 C	ND	46.978±0.087a	50.567±4.257a	46.978±0.087a	97.544±4.327a
T158 L	ND	25.797±0.602c	35.160±6.076bc	25.797±0.602c	60.957±6.626b
T158 M	ND	30.283±0.293b	33.756±3.697cd	30.283±0.293b	64.039±3.818b
T158 H	ND	20.992± 0.126d	25.788± 1.176de	20.992±0.126d	46.779±1.059c
Tam204 C	ND	25.418±0.318c	37.458±5.842bc	25.418±0.318c	62.877±5.644b
Tam204 L	ND	17.581±0.114de	44.113±3.571ab	17.581±0.114de	61.694±3.465b
Tam204 M	ND	16.688±1.257e	15.826±1.365f	16.688±1.257e	32.513±2.313de
Tam204 H	ND	19.010±0.265de	21.770±1.121ef	19.010±0.265de	40.780±0.880cd
4-Hydroxybenzoic acid					
Byrd C	0.601±0.006bc	2.009±0.028f	3.975±0.431a	2.610±0.034d	6.586±0.450abc
Byrd L	0.471±0.027de	1.773±0.008h	3.964±0.247a	2.244±0.025e	6.208±0.264bcd
Byrd M	0.426±0.0188efg	1.640±0.006i	3.743±0.337ab	2.065±0.017f	5.808±0.333d
Byrd H	0.462±0.0429ef	1.419±0.005j	3.253±0.044bcd	1.881±0.044g	5.135±0.043e
T158 C	0.536±0.003cd	2.580±0.047b	3.600±0.075abc	3.117±0.046b	6.717±0.070ab
T158 L	0.410±0.027efgh	2.214±0.013e	3.414±0.277abc	2.624±0.033d	6.038±0.297cd

T158 M	0.371±0.008gh	2.189±0.012e	3.392±0.177abc	2.560±0.014d	5.952±0.191d
T158 H	0.351±0.002h	1.930±0.025g	2.757±0.032d	2.281±0.027e	5.038±0.057e
Tam204 C	0.631±0.050b	3.343±0.020a	3.085±0.081cd	3.974±0.047a	7.059±0.128a
Tam204 L	0.551 ±0.010c	2.367±0.020d	2.718±0.012d	2.918±0.025c	5.636±0.019de
Tam204 M	0.824±0.006a	2.358±0.019d	2.733±0.023d	3.183±0.019b	5.916±0.040d
Tam204 H	0.397±0.006fgh	2.470±0.020c	2.782±0.013d	2.867±0.019c	5.648±0.030de
Vanillic acid					
Byrd C	1.503±0.114a	9.493±0.030a	6.859±0.107cd	10.996±0.138a	17.855±0.177bc
Byrd L	0.437±0.003cde	7.929±0.009e	7.970±1.278bc	8.366±0.009ef	16.336±1.273de
Byrd M	0.405±0.005cde	7.559±0.023f	6.539±0.562d	7.963±0.019g	14.503±0.575fg
Byrd H	0.374±0.012cde	6.757±0.028h	5.213±0.084e	7.130±0.033h	12.343±0.052h
T158 C	1.111±0.062b	9.483±0.055a	9.887±0.192a	10.594±0.073b	20.480±0.229a
T158 L	0.486±0.027cd	8.503±0.083c	9.214±0.255ab	8.990±0.080d	18.203±0.288bc
T158 M	0.343±0.007de	8.191±0.052d	9.838±0.075a	8.535±0.053e	18.373±0.125b
T158 H	0.331±0.006e	7.948±0.029e	7.373±0.072cd	8.279±0.034f	15.652±0.069ef
Tam204 C	1.166±0.110b	8.670±0.099b	7.176±0.082cd	9.835±0.106c	17.011±0.177cd
Tam204 L	0.494±0.017c	6.771±0.030h	7.274± 0.030cd	7.265±0.030h	14.540±0.033fg
Tam20 M	0.392±0.020cde	6.700±0.020h	7.009±0.077cd	7.092±0.041h	14.100±0.112g
Tam204 H	0.330±0.007e	6.954±0.042g	7.143±0.184cd	7.283±0.038h	14.427±0.152fg
Syringic acid					
Byrd C	1.947±0.262b	13.133±0.023bcd	3.778±0.891ab	15.080±0.330ab	18.858±0.561bc
Byrd L	1.895±0.022bc	14.108±0.182ab	5.557±1.510a	16.003±0.169a	21.578±1.352a
Byrd M	1.598±0.020bcde	6.084±0.144defg	3.383±0.106b	9.682±0.160def	13.066±0.256def
Byrd H	1.005±0.054f	6.124±0.130fgh	2.653±0.630b	7.128±0.105fg	9.781±0.673fg
T158 C	1.391±0.126def	14.550±0.330a	3.836±0.491a	15.941±0.296a	19.777±0.724ab

T158 L	1.746±0.085bcd	9.531±0.064cde	2.817±0.175b	11.277±0.041bcd	14.095±0.482cde
T158 M	1.298±0.089ef	8.483±0.175defg	3.345±0.095b	9.782±0.227cdef	13.127±1.083def
T158 H	0.594±0.136g	5.339±0.095gh	1.934±0.519b	5.933±0.205g	7.867±0.902g
Tam204 C	1.360±0.233def	12.332±0.130abc	2.538±0.635ab	13.693±0.354ab	16.230±0.931bcd
Tam204 L	1.623±0.073bcde	8.806±0.063def	2.091±0.390b	10.431±0.019cde	12.523±0.410ef
Tam204 M	1.519±0.154cde	7.289±0.016efgh	2.231±0.278b	8.808±0.140defg	11.040±0.149efg
Tam204 H	2.482±0.024a	4.846±0.056h	2.240±0.151b	7.328±0.073efg	9.569±0.089fg

Results are expressed as mean ± SD, n= 3. For each property across all varieties, values with different letters are significantly different (p<0.05). C-control group (falling number 550±40 seconds), L-low sprouting level (falling number 350±40 seconds), M-medium sprouting level (falling number 250±40 seconds), H-high sprouting level (falling numbers 120±40 seconds).

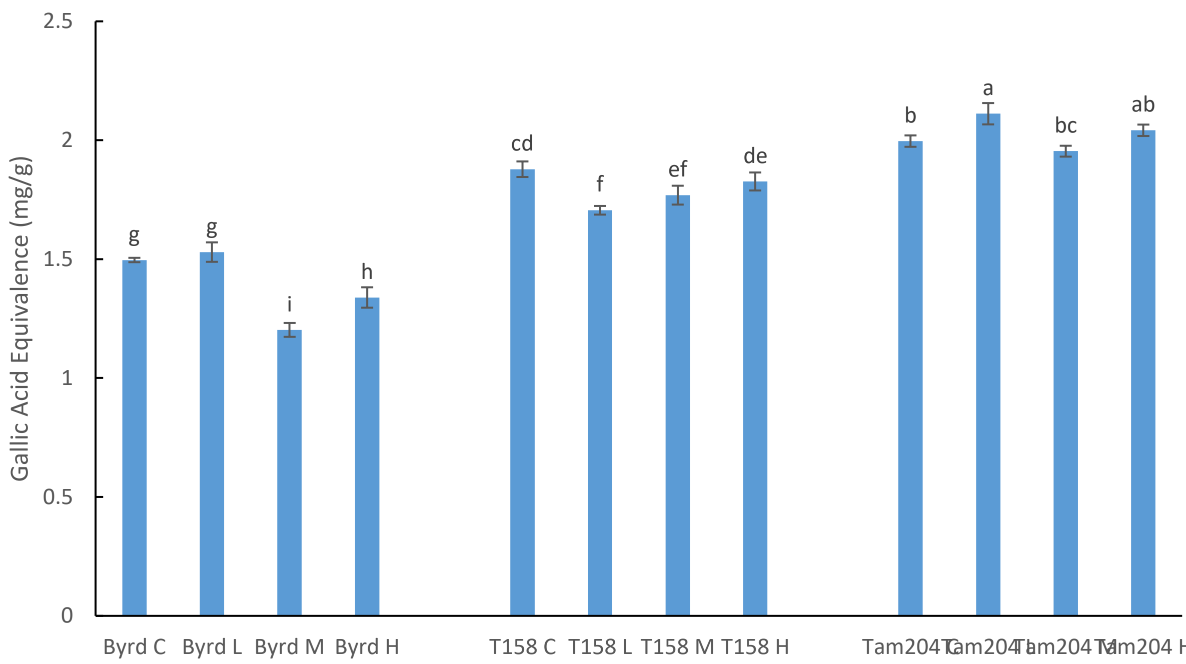

Figure 5-1 Total phenolic content of whole wheat flour samples.

Results are expressed as mean ± SD, n= 3. Across all varieties, bars with different letters are significantly different (p<0.05). C-control group (falling number 550±40 seconds), L-low sprouting level (falling number 350±40 seconds), M-medium sprouting level (falling number 250±40 seconds), H-high sprouting level (falling numbers 120±40 seconds).

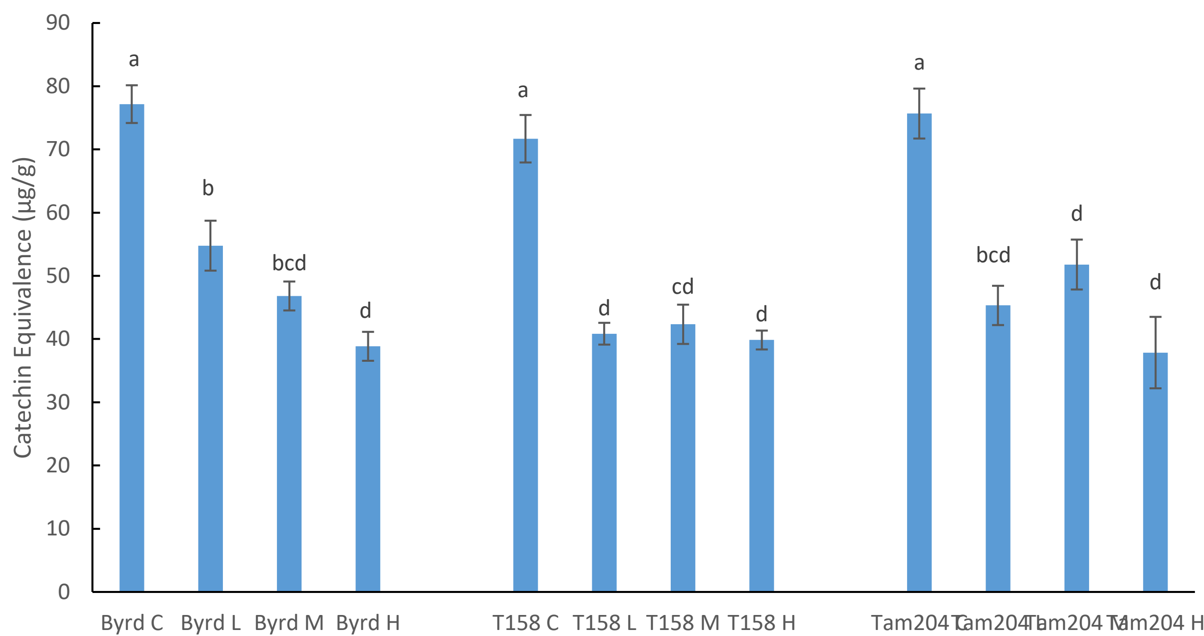

Figure 5-2 Total flavonoid content of whole wheat flour samples.

Results are expressed as mean ± SD, n= 3. Across all varieties, bars with different letters are significantly different ($p<0.05$). C-control group (falling number 550±40 seconds), L-low sprouting level (falling number 350±40 seconds), M-medium sprouting level (falling number 250±40 seconds), H-high sprouting level (falling numbers 120±40 seconds).

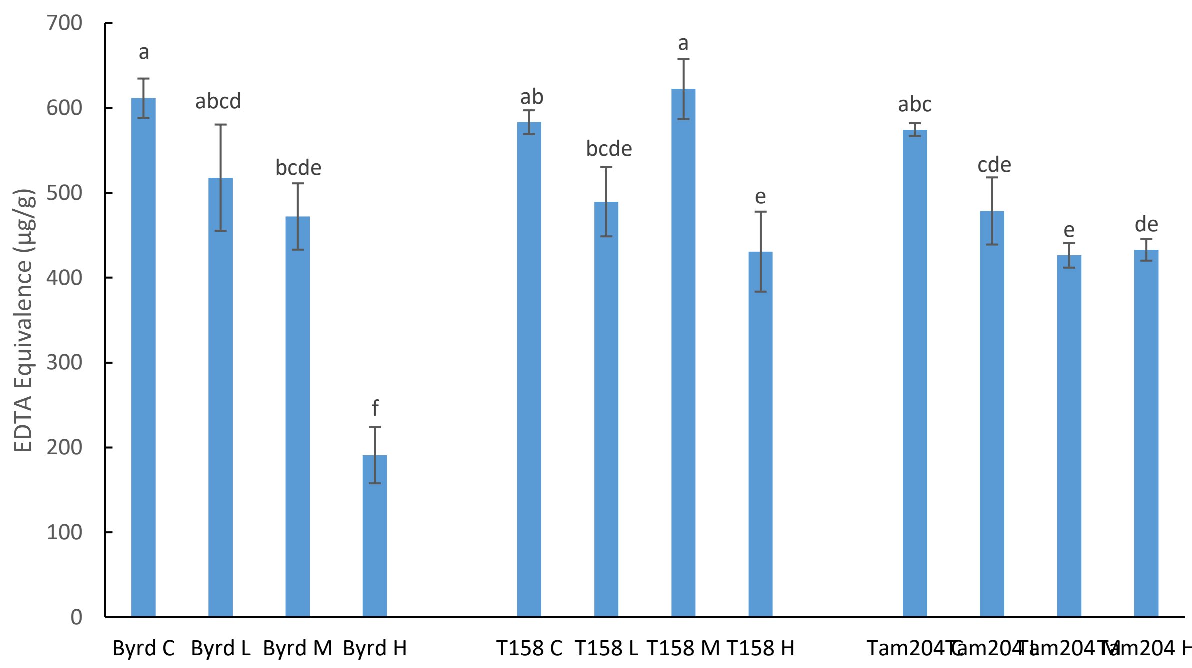

Figure 5-3 Metal chelating activity of whole wheat flour samples.

Results are expressed as mean ± SD, n= 3. Across all varieties, bars with different letters are significantly different ($p<0.05$). C-control group (falling number 550±40 seconds), L-low sprouting level (falling number 350±40 seconds), M-medium sprouting level (falling number 250±40 seconds), H-high sprouting level (falling numbers 120±40 seconds).

Chapter 6 - Changes in phenolic profiles and antioxidant activities during the whole wheat bread-making process*

Abstract

Health benefits of whole wheat products are partially attributed to their unique phenolic profiles. This study investigated the effect of bread-making processes on the phenolic profiles and antioxidant activities of four different varieties of hard red winter wheat. The fermentation process generally increased soluble phenolic content, flavonoid content, antioxidant activities, and soluble ferulic acid of whole wheat products. The baking process increased the soluble phenolic content and antioxidant activities. Some phenolic acids were incorporated into Maillard reaction products during baking. For the insoluble fraction, fermentation and baking slightly increased phenolic content, flavonoid content, and antioxidant activities of certain wheat varieties. Ferulic acid and isomers of di-ferulic acid (DFA) were not significantly affected by the baking process. Overall, the bread-making process demonstrated positive effects on the potential health benefits of whole wheat products.

Key words: wheat phenolics; antioxidant; fermentation; baking; wheat genotype; Maillard reaction

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6.1. Introduction

Epidemiological studies have demonstrated that consumption of whole wheat products may reduce the risk of chronic diseases such as cardiovascular disease, obesity, type II diabetes, and cancer (Jonnalagadda et al., 2011). The health benefits of whole wheat products can be attributed to their dietary fibers and phytochemicals (Liu, 2007). Wheat phytochemicals include phenolic acids, flavonoids, polyphenols, carotenoids, vitamin E, and others (Okarter & Liu, 2010). Phenolic compounds in wheat exist in soluble-free, soluble-conjugated, and insoluble-bound forms (Moore et al., 2005; Okarter et al., 2010). Ferulic acid, a strong natural antioxidant, is the most abundant simple phenolic acid in wheat (Adom et al., 2003; Graf, 1992). Phenolic profiles and antioxidant activities of whole wheat flours have been extensively studied (Liu et al., 2020). Total phenolic content (TPC), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), α, α-diphenyl-β-picrylhydrazyl (DPPH), and oxygen radical absorbance capacity (ORAC) assays are commonly used to evaluate the antioxidant actives. Phenolic acids were separated and quantified by reverse phase HPLC (Li et al., 2008; Okarter et al., 2010; Tian & Li, 2018).

Bread is an important processed wheat product that is consumed globally (Bachman et al., 2008). A typical bread-making process includes dough mixing, fermentation and proofing, and baking. These processes may result in changes of phenolic content, phenolic acid composition, and antioxidant activity of the final products. For example, thermal processing reduced the concentration of bio-active compounds compared to un-processed food ingredients in exotic fruits (Rawson et al., 2011). To fully take advantage of the potential health benefits of whole wheat products, it is crucial to understand the effect of processing such as bread-making steps on the phenolic profile and antioxidant activities.

Though some previous studies have described the effect of certain processing steps on wheat phenolics (Abdel-Aal & Rabalski, 2013; Gélinas & McKinnon, 2006; Yu et al., 2013; Yu & Beta, 2015), these studies often presented inconclusive or inconsistent results. The discrepancy may be due to comparison of data resulting from different wheat genotypes since the effect of processing may vary significantly among different wheat genotypes. The discrepancies may also be resulted from different bread-making protocols, phenolic extraction protocols, or experimental assays. Studies using different antioxidant assays may demonstrate different results for changes of antioxidant activities during bread-making steps, since there may not be positive correlations between different assays (Moore et al., 2005). To further understand phenolic changes during the bread-making, we investigated the changes of total phenolic content (TPC), total flavonoid content (TFC), ABTS, DPPH, and ORAC values, as well phenolic acid compositions including di-ferulic acids (DFA) of four different hard red winter wheat varieties (Ag Gallant, Tatanka, Turkey Red, and Zenda) with a wide range of protein content (8.9 - 15.0%). The objective of this study was to provide a comprehensive understanding on the effect of bread-making process on wheat phenolic profiles and antioxidant activities. Selection of different wheat varieties allowed us to understand if the responses to the bread-making processes would be different depending on the wheat genotypes. Our results bridge the knowledge gap of wheat antioxidants between raw materials and final food products.

6.2. Materials and methods

6.2.1. Reagents and chemicals

Standard phenolic acids (4-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, ferulic acid, sinapic acid, and gallic acid), Trolox, Folin-Ciocalteu reagent, DPPH and ABTS reagents, fluorescein, and 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Catechin standard, HPLC grade solvents, and other general chemicals were purchased from Fisher Scientific (Waltham, MA, USA). HPLC sample filter (0.45 μm) was purchased from Millipore Sigma (Burlington, MA, USA).

6.2.2. Wheat sample preparation and bread-making

Bread products are commonly produced using hard red wheats in the U.S. Four hard red winter wheat varieties (Zenda, protein content of 8.9%; Tatanka, protein content of 10.4%; Ag Gallant, protein content of 12.8%; and Turkey Red, protein content of 15.0%) were obtained from local seed companies. Grains (2 kg each) were first tempered to a final moisture content of 15% and then milled using a Quadrumat Senior mill (C.W. Brabender, South Hackensack, NJ, USA) according to the AACC Approved Method 26-50.01. The yield of refined flour was approximately 70%. The bran and shorts fractions were combined and re-mixed with the refined flour to obtain the whole wheat flour.

Bread was prepared according to the AACC Approved Method 10-10.03. The bread formulation included: 100 g whole wheat flour, 2.0 g yeast, 6.0 g sucrose, 1.5 g salt, 3.0 g shortening, and 0.2 g malt flour. For each flour sample, optimum water absorption and mixing time were determined

based on mixograph test. A 90-min fermentation and 33-min proofing times were used. After the proofing step, doughs were baked at 215 °C for 24 min to obtain breads.

Two independent dough samples were prepared from each wheat variety for each bread-making process: "mixing", "fermentation" (including both fermentation and proofing steps as described above), and "baking". The "mixing" and "fermentation" dough samples were immediately frozen at -20 °C after preparation. After baking, for each variety, two bread loaves were sliced, and bread crust and crumb were separated and frozen at -20 °C. Two additional loaves were also sliced and frozen, which were referred as the "bread loaf". The original bakery mixture that contained the same amount of flour, yeast, sucrose, salt, and shortening except for water was referred as the "flour" sample. The collected frozen samples and formulated flour were lyophilized, ground to fine powder, and kept at -20 °C freezer before analysis.

6.2.3. Extraction of soluble phenolic compounds

Soluble phenolic compounds were extracted according to methods described in a previous report (Okarter et al., 2010) with some modifications. In brief, five grams of each sample were mixed with 25 mL of 80% (v/v) acetone for 10 min. The mixture was centrifuged at 3500 xg for 15 min. The supernatant was collected, and the remaining residual was extracted again with another 25 mL of 80% acetone. After centrifugation, the supernatants were combined, evaporated to dryness using a rotary evaporator, and reconstituted with HPLC grade methanol to a final volume of 5 mL. The solution was filtered through a 0.45 µm filter, kept at -20 °C, and analyzed within 3 days. The solution was used for the measurements of soluble TPC, TFC, antioxidant activities, and soluble free phenolic acids.

To prepare HPLC samples for analysis of soluble-conjugated phenolic acids, the soluble extract (1 mL) was digested with 1 mL 4M NaOH for 3 hours under nitrogen atmosphere. The mixture was acidified with 1 mL 4M HCl and then extracted with 5 mL ethyl acetate for 3 times. The organic phase after each extraction was combined, evaporated to dryness, and reconstituted with 2 ml HPLC grade methanol. The product was filtered through a 0.45 μm filter and used for HPLC analysis. The quantification results of soluble-free and soluble-conjugated phenolic acids were combined and referred as the total soluble phenolic acids.

6.2.4. Extraction of insoluble phenolic compounds

Insoluble phenolic compounds were extracted according to our previous study (Tian & Li, 2018). Briefly, 1 g sample was first extracted with 80% acetone twice to remove the soluble fraction. The pellet was hydrolyzed with 10 mL 2 M NaOH solution for 3 hours under nitrogen. The mixture was acidified to pH 2 and centrifuged at 4000 x*g* for 15 min. The supernatant was extracted 3 times with ethyl acetate. The organic phases were combined and evaporated to dryness. The residue was reconstituted with 3 mL HPLC grade methanol, filtered through a 0.45 μm filter, kept at -20 °C, and analyzed within 3 days.

6.2.5. Phenolic acid analysis

Simple phenolic acids commonly found in whole wheat were identified and quantified by reverse phase HPLC using a Phenomenex Kinetex F5 column (Torrance, CA, USA) on an Agilent 1100 HPLC system equipped with a diode array detector (DAD) (Santa Clara, CA, USA). The mobile phase A was HPLC-grade water containing 0.1% trifluoroacetic acid (TFA), and mobile phase B was acetonitrile. The gradient program was reported previously (Tian et al., 2019). In brief,

mobile phase B was kept at 10% from 0-10 min, increased linearly from 10 to 20% from 10 to 17 min, and kept at 20% for another 5 min. Commercial phenolic acids were used as external standards for identification and quantification.

The UPLC-DAD-ESI-Q-TOF-MS/MS system from Waters Corporation (Milford, MA, USA) was used to further separate and identify the DFA isomers. The mobile phase A was LC-MS grade water containing 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. In brief, mobile phase B was kept at 9% from 0 to 1.2 min, increased linearly from 9 to 16% from 1.2 to 3.2 min, increased linearly from 16 to 19% from 3.2 to 4.9 min, increased linearly from 19 to 21% from 4.9 to 6.5 min, and kept at 21% for another 1 min. Flow rate of the mobile phase was 0.4 ml per min. The CORTECS UPLC C18 column was re-equilibrated for 2.5 min between each injection. The mass spectra was collected using a proprietary MS^E method provided by Waters Corporation with the following parameters: capillary voltage,2.53 kV; cone voltage, 25 V; de-solvation gas (N_2) flow, 600 L/h; cone gas (N_2) flow, 50 L/h; de-solvation temperature, 250 °C; ion source temperature, 100 °C. The ramp collision energy was set at 15 to 30V. The MS^E method recorded exact mass data for every detectable compound and its sub-structure simultaneously without discrimination and pre-selection. Isomers of DFA were tentatively identified according to the MS/ MS-MS spectra and relative retention times reported by previous studies using similar gradient protocols (Dobberstein & Bunzel, 2010; Gong et al., 2019).

6.2.6. Total phenolic content assay

The total phenolic content (TPC) was determined using the conventional colorimetric method (Singleton & Rossi, 1965). In brief, the sample extract (0.1 mL) was mixed with 7.9 mL

deionized (DI) water and then with 0.5 mL Folin-Ciocalteu reagent. After 5 min, 1.5 mL 20% Na_2CO_3 solution was added to the mixture. After color development for 2 hours, the absorbance at 765 nm was recorded using a VWR UV1600-PC spectrophotometer (Radnor, PA, USA). Gallic acid was used as a standard to establish the calibration curve. The final TPC value was expressed as microgram gallic acid equivalence (GAE) per gram of the tested sample (μg GAE/ g).

6.2.7. Total flavonoid content assay

The total flavonoid content (TFC) was determined according to a previous report (Chen & Chen, 2011). In brief, 0.5 mL sample extract was mixed with 3.2 mL deionized (DI) water and 0.15 mL 5% $NaNO_2$ solution. After 5 min, 0.15 mL 10% aluminum chloride ($AlCl_3$) solution was added. After an additional 1 min, 1 mL 1M NaOH was added to the mixture. The absorbance at 510 nm was measured using a VWR UV1600-PC spectrophotometer (Radnor, PA, USA). A calibration curve was established using catechin as the standard. The final TFC was expressed as microgram catechin equivalence (CE) per gram of the tested sample (μg CE/ g).

6.2.8. ABTS radical scavenging assay

The ABTS radical scavenging activity was determined according to our previous study (Tian et al., 2019). In the dark, 50 μL sample extract was reacted with 3.9 mL 0.13 mM ABTS for 10 min. Then, the absorbance at 734 nm was measured using a VWR UV1600-PC spectrophotometer. The ABTS value was expressed as micromole Trolox equivalence per gram of sample (μmol TE/ g).

6.2.9. DPPH radical scavenging activity assay

The DPPH free radical scavenging activity was determined following a method reported previously (Villaño et al., 2007). Sample extract (50 µL) was reacted with 3.9 mL DPPH solution in the dark. After 25 min, the mixture was centrifuged at 3000 x*g* for 5 min, and then the absorbance at 517 nm was recorded. The DPPH value was expressed as micromole Trolox equivalence per gram of sample (µmol TE/ g).

6.2.10. Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was performed according to a method reported previously (Zulueta et al., 2009). Fluorescein (FL) was used as the fluorescent probe. In brief, diluted sample extract (50 µL) was mixed with 6 nM FL solution (100 µL) in a 96-plate microplate. The mixture was incubated in a Biotek Synergy H1 Hybrid microplate reader (Winooski, VT, USA) at 37 °C for 30 min. Then, 76.5 mM AAPH solution (50 µL) was added to the mixture. The excitation and emission wavelengths were 485 and 528 nm, respectively. The intensity of the fluorescence was recorded every min for 2 hours at 37 °C. The net area under the curve (AUC) of the tested sample was calculated using the following equation:

$AUC = 0.5 + F_1/F_0 + F_2/F_0 + \ldots F_i/F_0 \ldots + F_{119}/F_0 + 0.5*F_{120}/F_0$, where F_i = fluorescence reading at min i.

The final ORAC value was quantified using a Trolox standard curve and expressed as micromole Trolox equivalence per gram of sample (µmol TE/ g).

6.2.11. Statistical analysis

The results were reported as mean ± standard deviation (SD) from four replicates. Results were subjected to one-way analysis of variance (AVONA) and Turkey's test using SAS software, version 9.3 (Cary, NC, USA). $P < 0.05$ was considered as significantly different.

6.3. Results and discussions

6.3.1. Total phenolic content and total flavonoid content

Table 6-1 summarizes the total phenolic content (TPC) and total flavonoid content (TFC) of the tested samples. The soluble and insoluble fractions are presented separately. For the soluble fraction of all the four wheat varieties, the fermentation process significantly ($p< 0.05$) increased TPC values by 11.4%, 9.0%, 5.9% and 7.5%, respectively, compared to the corresponding mixing dough samples. For Ag Gallant and Turkey Red, mixing dough samples also had significantly higher ($p< 0.05$) TPC values than the flour samples. Bread crust exhibited the highest TPC among all the tested samples, partially due to Maillard reaction products that reacted with the Folin-Ciocalteu reagent (Gélinas & McKinnon, 2006; Michalska et al., 2008). Except for Ag Gallant, the TPC of bread crumb was also significantly higher ($p< 0.05$) than the corresponding TPC of the fermentation sample. This suggested the positive effect of thermal processing on the release of soluble phenolics. A similar effect was also reported in previous studies (Călinoiu & Vodnar, 2020; Lu et al., 2014). Overall, these results suggested the positive effect of thermal processing on soluble phenolic content.

Yu & Beta (2015) also found that soluble TPC increased after fermentation and attributed this phenomenon to the release of insoluble-bound phenolics. However, our study did not show a

significant decrease of insoluble-bound TPC after fermentation. Some soluble phenolic compounds may exist in high- molecular-weight (HMW) polyphenol complexes with proteins or carbohydrates. We speculated that during the mixing and fermentation process, the HMW polyphenol networks were partially broken down to relatively low-molecular-weight (LMW) polyphenols and therefore became more accessible to the Folin- Ciocalteu reagent during analysis. Similarly, thermal processing may also partially break down the soluble HMW polyphenols. Thus, the bread crumb generally had higher TPC than the fermentation dough.

In terms of insoluble bound fractions, generally, TPC values were not significantly changed after dough mixing and fermentation. Except for Tatanka, the bread loaf demonstrated significantly higher ($p< 0.05$) TPC values than the corresponding fermentation dough, which is in agreement with the report by Yu & Beta (2015). Thermal processing may further release some phenolic compounds that cannot be liberated by conventional alkaline hydrolysis. In our study, bread crumb and bread crust overall had very close insoluble-TPC values, implying that the Maillard reaction products may only exist in the soluble fractions.

Changes of soluble total flavonoid content (TFC) during the bread-making steps were similar to that of the soluble TPC. Compared to the flour sample, mixing and fermentation process generally increased soluble TFCs of the samples. Bread crust had the highest soluble TFC value, possibly due to interference from Maillard reaction products (MRPs). For the insoluble- bound fraction, mixing and fermentation generally did not influence the TFC values of the tested varieties. For Tatanka, Turkey Red, and Zenda, TFCs of bread loaf samples increased by 19.3, 15.8, and 34.2%, respectively, compared to the fermentation dough. It seemed that thermal

processing had positive effect on the TFC values. This observation was in agreement with previous studies on the effects of thermal processing on flavonoid content of garlic, tomatoes, and beets (Dewanto et al., 2002; Jiratanan & Liu, 2004; Kim et al., 2013).

6.3.2. Free radical scavenging activities

Figure 6-1(a) and **Figure 6-1(b)** show the ABTS radical scavenging activity of soluble and insoluble-bound wheat phenolic extract, respectively. Similarly, **Figure 6-2(a)** and **Figure 6-2(b)**show the DPPH radical scavenging activity of the soluble and insoluble-bound wheat phenolic extract. **Figure 6-3(a)** and **Figure 6-3(b)**present the ORAC values of the soluble and insoluble-bound wheat phenolic extract.

The mixing process did not influence the soluble ABTS except for the Ag Gallant variety. After the fermentation process, soluble ABTS of the four tested varieties significantly increased ($p < 0.05$) by 24.8, 38.5, 31.4, and 22.4%, respectively, compared to the corresponding flour sample. Interestingly, for each variety, bread crumb always had significantly higher ($p< 0.05$) soluble ABTS value than the fermentation dough. This could provide evidence suggesting that besides the formation of MRPs, the simple thermal processing also liberated some soluble antioxidants. Bread crust samples showed higher ABTS values than bread loaf samples and crumb samples. In terms of the insoluble-bound fraction, the fermentation process slightly increased the ABTS value of Tatanka. For Ag Gallant and Turkey Red, the baking process slightly increased the ABTS values of bread loaf compared to the fermentation dough. Generally, ABTS values of the insoluble fraction were less affected by the entire bread-making process, compared to the soluble fraction.

For DPPH values of the soluble fraction, the mixing process increased the DPPH value of Ag Gallant by 108.2% but decreased the DPPH value of Tatanka by 14.0%. The mixing process did not significantly change the DPPH values of Turkey Red and Zenda, while the fermentation process increased the DPPH values by 20.3 and 26.7%, respectively, compared to the flour sample. The bread crust had the highest DPPH value within each wheat variety. For the insoluble-bound fraction, the baking process significantly ($p < 0.05$) increased the DPPH values of bread fractions of Turkey Red variety samples. For the other three varieties, there were no major changes of the insoluble-bound DPPH values during the entire bread-making process. Within each variety, bread crust had similar DPPH value to the bread loaf and bread crumb.

Though some changes were not significant ($p< 0.05$), the trend of change for the soluble ORAC values during the bread-making process was similar to that of soluble ABTS or DPPH. The baking process seemed to have some positive effects on the insoluble-bound ORAC values. The ORAC values of bread crumb of Ag Gallant and Zenda were significantly ($p< 0.05$) higher than the corresponding dough samples. Interestingly, the bread crust of Zenda had significantly lower ($p< 0.05$) ORAC value than its bread crumb. For Tatanka, the insoluble ORAC value did not change during the bread-making process. For Turkey Red, bread fractions showed higher ORAC values than the flour and dough samples. This was in agreement with the observation on insoluble DPPH values.

The effect of the bread-making processes on antioxidant activity of whole wheat products has been described to some extent in previous studies. Yu et al. (2013) reported that baking

decreased soluble DPPH value but increased soluble ORAC values of both whole flour and refined flour. Moore et al. (2009) reported that fermentation did not increase soluble ABTS, DPPH, and ORAC values while the baking process significantly increased these values in pizza crust. In general, our results showed that both fermentation and baking processes generally had positive effect on the antioxidant activity of the soluble fractions. This was in agreement with a previous study (Yu & Beta, 2015) that soluble TPC and antioxidant activities of purple kernel wheat flours significantly increased during the fermentation and baking process. Yu & Beta (2015) also found that the bread crust had significantly lower insoluble-bound DPPH and ABTS values than bread crumb; however, in our study, the bread crust and crumb had very close insoluble ABTS and DPPH values. In summary, the fermentation and baking process had an overall positive effect on soluble antioxidant activity. Results from ABTS, DPPH, and ABTS assays showed generally good correlations, but some discrepancies did exist. The mixing and fermentation process had no apparent effect while the baking process increased the antioxidant values for certain wheat varieties, implying that the antioxidant changes during the breadmaking process was also dependent on the intrinsic features of wheat genotypes.

6.3.3. Identification of di-ferulic acids

The 8-8’ DFA, 8-5’ DFA, 5-5’ DFA, 8-O-4’ DFA, and 8-5’ benzofuran DFA were common di-ferulic acids found in whole grains (Gong et al., 2019). DFAs exist in both soluble and insoluble-bound fractions. The theoretical $[M-H]^-$ (m/z) for DFA is 385.0929. In this study, 5 isomers of DFA were detected according to the $[M-H]^-$ (m/z) peak (**Figure 6-4**). Due to a lack of analytical standard, the isomers were identified according to their relative retention time as well as fragmentations from MS^E spectra (**Figure 6-5**). Several previous studies showed that isomers of

DFA were eluted in the order of 8-8’ DFA, 8-5’ DFA, 5-5’ DFA, 8-O-4’ DFA, and 8-5’ benzofuran DFA (Dobberstein & Bunzel, 2010; Gong et al., 2019; Guo & Beta, 2013). The peak at 5.138 min can be assigned as 5-5’DFA as it was reported that 5-5’ DFA had the highest intensity for the $[M-H]^-$ peak (Gong et al., 2017).The peak at 6.482 min were confirmed to be 8-O-4’ DFA due to its strongest m/z peak at 193.0506. The 8-O-4’ DFA was most likely to present ferulic acid monomer in the mass spectroscopy (Gong et al., 2019). The peak at 3.627 min was assigned as 8-5’ DFA due to its strong fragmentation m/z at 267.0665 $[M-H-CO_2 \times 2-CH_3 \times 2]^-$. The peak at 6.906 min was assigned as 8-5’ benzofuran DFA according to the presence of peaks including 341.1056 ($[M-H-CO_2]^-$), 326.0812 ($[M-H-CO_2-CH_3]^-$), 282.0880 ($[M-H-CO_2 \times 2-CH_3]^-$), 267.0665 ($[M-H-CO_2 \times 2-CH_3 \times 2]^-$, and its longest retention time (Gong et al., 2017).

6.3.4. Composition of soluble phenolic acids

Soluble phenolic acids can exist in either soluble-free or soluble-conjugated fractions. Both fractions contributed to the soluble TPC and antioxidant activities; therefore, in this study soluble-free and soluble-conjugated phenolic acids were quantified and added up together as the total soluble phenolic acid. Common simple phenolic acids were identified by comparing the retention time with external standards (Tian & Li, 2018). **Table 6-2** shows composition of total soluble phenolic acids of the samples during the bread-making process. 4-hydroxybenzoic acid, syringic acid, vanillic acid, *p*-coumaric acid, ferulic acid, and sinapic acid were detected in the soluble fractions.

Soluble 4-hydroxybenzoic acid and *p*-coumaric acid were generally not influenced by the mixing and fermentation. Syringic acid greatly decreased during the fermentation process by 45.3, 78.2, and 76.2% for Ag Gallant, Tatanka, and Zenda, respectively. This result suggested that syringic acid might be involved in the metabolism of the yeast during fermentation. Differently, the mixing and fermentation greatly increased soluble ferulic acid by 63.0, 41.1, 66.2, and 100.8%, respectively, for the tested varieties. This observation agreed with some previous reports (Abdel-Aal & Rabalski, 2013; Yu & Beta, 2015). Similarly, the fermentation process significantly ($p < 0.05$) increased soluble vanillic acid of Ag Gallant and Tatanka. The effect of fermentation on sinapic acid was highly dependent on wheat variety. The fermentation process greatly decreased sinapic acid of Ag Gallant by 62.4%, but to some extent increased sinapic acid of other wheat varieties. In summary, the effect of mixing and fermentation was complicated and dependent on both wheat varieties and type of the phenolic acid. During the fermentation process, yeast activity may partially degrade the phenolic acid-carbohydrate complex and therefore increase the amount of soluble phenolic acids. On the other hand, yeast metabolism activity may consume phenolic acids or transfer certain phenolic acid to other type of phenolic acid or other compounds.

In terms of the baking process, bread loaf and fermentation dough of each variety demonstrated almost identical phenolic acid composition. However, the baking process greatly influenced the distribution of phenolic acids (**1**). It can be found that the bread crust generally contained lower level of major phenolic acids than the bread crumb. For example, for Ag Gallant, syringic acid, vanillic acid and ferulic acid of bread crust and bread crumb were 5.97 and 12.41, 18.08 and 23.57, and 28.63 and 37.27 μg/g, respectively. For Zenda, syringic acid, vanillic acid and ferulic

acid of bread crust and bread crumb were 0.86 and 1.73, 6.12 and 9.34, and 36.22 and 51.63 μg/g, respectively. Pérez-Jiménez et al. (2014) also found that bread crust had significantly lower ferulic acid content than bread crumb. We hypothesized that some wheat phenolic acids, such as ferulic acid, might be incorporated into MRPs during baking. UPLC-MS spectra of fermentation dough, bread crumb, and crust samples (**Figure 6-6**) provided evidence supporting this hypothesis. The bread crust showed an extra peak compared to the bread crumb or fermentation dough at 2.30 min. The detailed fragmentation of this peak was provided in **Figure 6-7**. The m/z at 193.05 was characteristic for ferulic acid and the m/z at 174.0563 suggested an elemental composition of $C_{10}H_9NO_2$ which can possibly be N-furfuryl-2-formylpyrrole, a 2-formylpyrrole derivative from MRPs (Wood et al., 2019) and a contributor to popcorn aroma (Buttery et al., 1997; Shen & Hoseney, 1995). Among the four varieties, the decrease of soluble ferulic acid was positively correlated (R^2=0.89) with the increase of intensity of m/z of 193.05 at 2.30 min (**Figure 6-8**), which further confirmed the incorporation of soluble ferulic acid into MRPs. However, some exceptions existed. For example, for Turkey Red, the bread crust contained more 4-hydroxybenzoic acid than the bread crumb; for Ag Gallant, the bread crust contained more sinapic acid than the bread crumb. This might be because that the thermal processing can also help release some phenolic acid from polyphenol-carbohydrate complex. During the baking process, incorporation and release of phenolic acids can occur simultaneously (Alves & Perrone, 2015). The combined effect led to the conclusion that the whole wheat products from each variety had similar phenolic acid composition before and after baking but bread crust and crumb had significantly different phenolic acid compositions.

The effects of bread-making steps on the composition of soluble DFAs also varied among different wheat varieties. Generally, the mixing process increased the content of soluble DFAs especially for 5-5’ DFA, 8-O-4’ DFA and 8-5’ benzofuran DFA. The baking process increased content of 8-5’ DFA but decreased content of 8-5’ benzofuran DFA. Content of 8-8’ DFA remained relatively constant during the bread-making process (**Figure 6-9**).

6.3.5. Composition of insoluble-bound phenolic acids

Table 6-3 shows the composition of total insoluble-bound phenolic acids of the samples during the bread-making process. The amount of ferulic acid and sinapic acid ranged from 278.4 to 488.9 μg/ g and 8.6 to 159.0 μg/ g, respectively. Other simple phenolic acids were detected but the amount was much lower compared to the amount of ferulic acid or sinapic acid. Changes in the DFA contents during the bread-making process were also evaluated according to the changes of peak area (m/z= 385.09) but not quantified due to a lack of analytical standards.

The mixing process significantly ($p< 0.05$) decreased the content of sinapic acid for Ag Gallant and Turkey Red by 65.5% and 35.5%, respectively. The amount of insoluble ferulic acid was generally not influenced by the bread-making process. This was in agreement with a previous study which reported that the baking process did not decrease insoluble phenolic acids (Mattila et al., 2005). Lu et al. (2014) reported that fermentation had little effect and the baking process slightly increased the amount of ferulic acid and total phenolic acid. However, Abdel-Aal & Rabalski (2013) reported that insoluble ferulic acid decreased after the baking process. Though no significant change of ferulic acid was observed at $p< 0.05$ in our study, generally, the baking process increased ferulic acid content of Tatanka and Turkey Red but decreased that of Ag

Gallant. Therefore, it seemed that changes of ferulic acid during the baking process were dependent on wheat varieties. Changes of DFAs also seemed to be dependent on wheat varieties. For Ag Gallant, the baking process increased the content of several DFA isomers especially for 5-5' DFA (**Figure 6-10**). For other tested varieties, DFA composition was not significantly influenced by the bread-making process. This is perhaps the reason for inconsistent results reported in the literature. However, our results and previous reports generally agree that insoluble phenolic acids as well as antioxidant activity were well preserved after the fermentation and baking of whole wheat bread.

6.4. Conclusions

In this study, we reported changes of total phenolic content (TPC), total flavonoid content (TFC), antioxidant activities, and phenolic acids composition of four hard red winter wheat varieties during the bread-making process. The bread-making process generally had positive effect on phenolic profile and antioxidant activities of whole wheat products. For soluble fractions, bread crust demonstrated the highest antioxidant values due to the contribution of MRPs. The fermentation process consumed some syringic acid but increased the amount of vanillic acid and ferulic acid. Some phenolic acids were incorporated into Maillard reaction products during baking. Comparing to soluble fractions, insoluble fractions were relatively less affected by the fermentation and baking process. These changes were also dependent on wheat varieties, to some extent. In summary, our study demonstrated that bread-making process may enhance potential health benefits of whole wheat products. The interaction effect from wheat variety is another important factor that should be considered. Further studies are necessary to optimize the

processing conditions and selection of wheat varieties for bakery products with potentially improved health benefits.

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Conflict of interest

The authors declare no conflicts of interest.

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Table 6-1 Total phenolic content (TPC) and total flavonoid content (TFC) of wheat varieties at different stages of bread-making process.

	Ag Gallant	Tatanka	Turkey Red	Zenda	Ag Gallant	Tatanka	Turkey Red	Zenda
	Soluble TPC (μg GAE/ g)				**Insoluble-TPC (μg GAE/ g)**			
Flour	470.1± 28.09e	392.46 ± 1.74e	322.45± 4.03f	211.55± 5.00e	1201.64± 20.16c	1134.82± 14.75a	1278.68± 32.53b	1393.27± 57.25b
Mixing	503.82± 2.35de	401.09 ± 8.66e	346.09± 3.11e	224.73± 24.35de	1051.64± 11.35d	1074.16± 16.06b	1273.23± 7.51b	1406.86± 43.88b
Fermentation	561.09± 20.08bc	437.00 ± 15.53d	366.55± 3.32d	241.55± 9.78d	1173.0± 22.32c	1125.95± 23.09ab	1251.41± 44.33b	1412.32± 61.81b
Bread Loaf	582.45± 4.55b	517.00 ± 9.89b	579.27± 3.93b	378.82± 3.74b	1346.18± 10.20a	1122.55± 17.25ab	1378.91± 22.60a	1537.09± 25.68a
Bread Crust	882.00± 16.09a	767.91± 12.00a	1016.10± 11.34a	628.82± 15.67a	1343.45± 10.20a	1143.33± 33.59a	1365.27± 38.11a	1419.82± 31.18b
Bread Crumb	539.27± 6.64cd	480.64± 4.03c	427.46±4.81c	279.72± 4.78c	1283.45± 13.73b	1176.41± 27.30a	1384.36± 7.04a	1488.68± 38.59ab
	Soluble TFC (μg CE/ g)				**Insoluble-TFC (μg CE/ g)**			
Flour	48.78± 3.91e	37.85± 1.64c	36.46± 1.85d	46.83± 1.43bc	83.28± 3.19c	78.28± 5.16c	110.77± 6.85c	74.39± 7.98c
Mixing	51.19± 2.02de	37.29± 2.07c	39.98± 2.78cd	42.82± 4.13c	76.61± 9.49c	87.44± 4.80bc	120.50± 1.06b	88.28± 3.89bc
Fermentation	56.74± 0.48cd	55.54± 5.99a	45.63± 0.98c	52.94± 4.00b	85.78± 4.58bc	84.94± 9.40bc	121.61± 1.40b	99.94± 7.33bc
Bread Loaf	64.89± 3.45ab	50.72± 0.63ab	63.59± 6.56b	52.02± 3.00b	96.89± 6.70ab	101.33± 5.33a	140.78± 3.74a	134.11± 7.43a
Bread Crust	70.72± 2.14a	57.11± 4.07a	85.81± 1.91a	70.44± 6.42a	101.06± 4.75a	96.89± 2.80ab	142.17± 1.40a	127.17± 3.06ab
Bread Crumb	60.82± 4.34bc	48.04± 1.09b	46.83± 1.67c	51.74± 2.72b	96.61± 4.10ab	101.33± 4.67a	137.72± 5.84a	126.89± 3.21a

Results are expressed as mean ± std, n=3. TPC is expressed as μg gallic acid equivalence (GAE) per gram sample (μg GAE/ g). TFC is expressed as μg catechin equivalence (CE) per gram sample (μg CE/ g). Within each variety, numbers with no letter in common are considered to be significantly different ($p < 0.05$).

Table 6-2 Composition of total soluble phenolic acids of wheat varieties at different stages of bread-making process.

		4-Hydroxybenzoic acid (µg/g)	Syringic acid (µg/g)	Vanillic acid (µg/g)	*p*-Coumraic-acid (µg/g)	Ferulic acid (µg/g)	Sinapic acid (µg/g)
Ag Gallant	Flour	3.37± 0.36c	20.29± 4.94b	9.61± 0.55c	10.22± 0.13cd	19.08±0.39 d	19.66± 0.64a
	Mixing	5.34± 0.24a	27.31± 0.16a	16.21± 0.23b	11.52± 0.16a	31.07± 1.84bc	7.40± 0.18c
	Fermentation	5.06± 0.22a	14.93±0.78 c	23.78± 1.86a	10.63± 0.06bc	32.04±1.98 b	7.08± 0.93c
	Bread loaf	4.38± 0.28b	12.22± 0.30c	24.21± 1.36a	11.40± 0.27a	33.69±0.73 b	8.00± 0.17c
	Bread crust	3.26± 0.08c	5.97± 0.51d	18.08±0.43b	9.89± 0.47d	28.63±0.94 c	15.03± 2.21b
	Bread crumb	4.26± 0.26b	12.41± 0.14c	23.57± 0.11a	11.04± 0.15ab	37.27± 0.29a	6.31± 0.51c
Tatanka	Flour	8.83± 0.42a	15.05± 0.96a	6.37± 0.83c	8.30± 0.18a	19.51± 1.26d	21.39± 0.68bc
	Mixing	8.61±0.37a	13.92± 0.67a	7.42± 0.35bc	8.58± 0.39a	22.78± 3.24cd	21.57± 2.07b
	Fermentation	10.34± 1.41a	3.03± 0.37b	9.00± 0.17a	8.51± 0.89a	27.52±0.79 a	26.26±1.20a
	Bread loaf	9.06± 0.97a	1.91± 0.14bc	8.49± 0.14ab	8.63±0.14 a	26.25± 0.67abc	17.84± 1.71d
	Bread crust	6.55± 0.85b	1.02± 0.16c	6.68± 0.83c	7.89± 0.93a	23.44± 1.65bc	18.38± 1.21cd
	Bread crumb	9.24± 0.10a	2.10± 0.06bc	9.15± 0.45a	8.45± 0.54a	26.89± 0.54ab	18.40± 0.90cd
Turkey Red	Flour	3.62± 0.05cd	11.80± 0.38a	9.21± 0.49cd	3.52± 0.15a	14.26± 1.42d	17.44± 2.80a
	Mixing	3.35± 0.13de	11.10± 1.14ab	8.17± 1.12d	3.61± 0.33a	17.82± 5.17cd	17.56± 6.10a

	Fermentation	2.96± 0.38e	9.83± 0.79b	10.58± 0.21bc	3.59± 0.20a	23.70± 6.25abc	21.47± 6.98a
	Bread loaf	5.69± 0.06b	2.53± 0.04cd	10.54± 0.32bc	3.03± 0.05b	27.21± 1.42ab	22.16± 1.47a
	Bread crust	10.71± 0.12a	1.63± 0.13d	11.14± 1.13ab	2.38± 0.01c	20.09± 2.57bcd	14.69± 4.84a
	Bread crumb	3.84± 0.12c	3.12± 0.10c	12.33± 0.36a	3.31± 0.08ab	30.69± 1.81a	23.93± 1.75a
Zenda	Flour	4.02 ± 0.40ab	20.39± 1.68a	7.16±1.03abc	nd	28.99± 2.66c	27.44± 1.03b
	Mixing	4.15± 0.19ab	19.87± 5.25a	6.80± 1.81bc	nd	42.09± 13.64abc	38.57±13.48ab
	Fermentation	2.62± 0.00b	4.73± 0.78b	9.06± 0.58ab	nd	58.20± 2.02a	49.88± 7.45a
	Bread loaf	3.94± 0.70ab	1.77± 0.14c	8.44± 0.92ab	nd	52.02± 7.03ab	42.04± 5.02ab
	Bread crust	4.97± 1.39a	0.86± 0.09d	6.12± 0.31c	nd	36.22± 6.88bc	25.10± 1.57b
	Bread crumb	3.75± 0.79ab	1.72± 0.13c	9.34± 0.73a	nd	51.63± 7.72ab	33.19± 10.34ab

Results are expressed as mean ± std, n=3. Phenolic acid was expressed as µg/g sample. For each phenolic acid of each variety, numbers with no letter in common are considered as significantly different ($p < 0.05$). nd: not detected.

Table 6-3 Composition of insoluble-bound phenolic acids of wheat varieties at different stages of bread-making process.

		4-Hydroxybenzoic acid (μg/g)	Syringic acid (μg/g)	Vanillic acid (μg/g)	*p*-Coumraic acid (μg/g)	Ferulic acid (μg/g)	Sinapic acid (μg/g)
Ag Gallant	Flour	5.88± 0.04a	1.31± 0.12a	16.85± 0.20ab	21.15± 2.49a	462.38± 40.39a	25.04± 7.30a
	Mixing	5.34± 0.11b	nd	13.96± 0.48d	20.29± 3.54a	440.722± 22.70a	8.74± 3.46b
	Fermentation	5.40± 0.12b	nd	14.51± 0.58cd	18.61± 5.23a	488.90± 80.24a	8.59±1.00b
	Bread loaf	5.65± 0.34ab	nd	16.17± 0.76abc	22.75± 0.44a	468.47± 50.28a	32.84± 2.74a
	Bread crust	6.00± 0.04a	nd	17.70± 1.96a	22.64± 2.64a	482.63± 99.32a	27.98± 2.59a
	Bread crumb	5.35± 0.14b	nd	14.98±0.08bcd	20.82± 1.71a	449.91± 57.06a	27.84± 5.98a
Tatanka	Flour	nd	2.27±0.07 ab	24.38± 6.15a	19.05± 0.11ab	284.27± 42.64a	79.38± 13.18a
	Mixing	nd	2.09± 0.12c	22.72± 6.73a	16.09± 3.26b	278.41± 50.38a	75.12± 13.31a
	Fermentation	nd	2.40± 0.19ab	23.27± 5.81a	19.45± 1.27ab	295.35±35.20a	84.28± 11.62a
	Bread loaf	nd	2.50± 0.20b	30.36± 6.93a	17.95± 0.25ab	295.53± 61.08a	93.77± 11.04a
	Bread crust	nd	2.39± 0.22ab	20.72± 2.66a	18.80±1.43ab	301.02± 11.34a	94.30± 7.26a
	Bread crumb	nd	2.96± 0.23a	29.07± 3.89a	20.16±1.86a	316.00± 16.98a	94.74±4.56a
Turkey Red	Flour	5.60± 0.20a	4.13± 0.77a	11.59± 1.42ab	28.43± 1.10a	358.16± 13.18ab	105.37± 10.28d
	Mixing	5.50± 0.08a	4.11± 0.74a	11.79± 1.09ab	27.63± 0.82a	354.16± 4.19ab	67.92± 0.90e
	Fermentation	5.47± 0.33a	nd	13.69± 1.37a	27.85±1.48a	346.43± 19.31ab	63.59± 1.91e

	Bread loaf	nd	4.39± 0.59a	8.42± 0.23c	25.70± 4.19a	361.41± 4.05a	158.96±6.40a
	Bread crust	nd	4.47± 0.40a	10.19± 0.60bc	27.76± 1.13a	334.99± 13.77b	130.89± 5.32c
	Bread crumb	nd	4.00± 0.18a	8.70± 0.17c	28.14± 0.87a	368.86± 1.58a	149.60± 6.47ab
Zenda	Flour	5.32± 0.13e	nd	22.15± 0.39a	40.71± 1.52b	412.20± 13.82ab	107.65± 14.31ab
	Mixing	8.65± 0.24a	nd	19.06± 0.32cd	41.36±3.36b	422.90± 28.45ab	86.21± 8.84b
	Fermentation	7.81± 0.79ab	nd	18.31± 0.92d	43.59± 2.95b	419.08± 2.83ab	95.43± 15.77ab
	Bread loaf	6.69± 0.10cd	nd	19.67± 0.48bc	46.29± 7.13ab	437.22± 17.12a	119.14± 12.86ab
	Bread crust	6.07± 0.11de	nd	20.41± 0.67b	48.89± 9.72ab	390.91± 1.91ab	84.84± 21.29b
	Bread crumb	7.43± 0.53bc	nd	19.06± 0.50cd	55.67±2.21a	415.83± 7.37b	124.28±20.28a

Results are expressed as mean ± std, n=3. Phenolic acid was expressed as μg/g sample. For each phenolic acid of each variety, numbers with no letter in common are considered as significantly different ($p < 0.05$). nd: not detected.

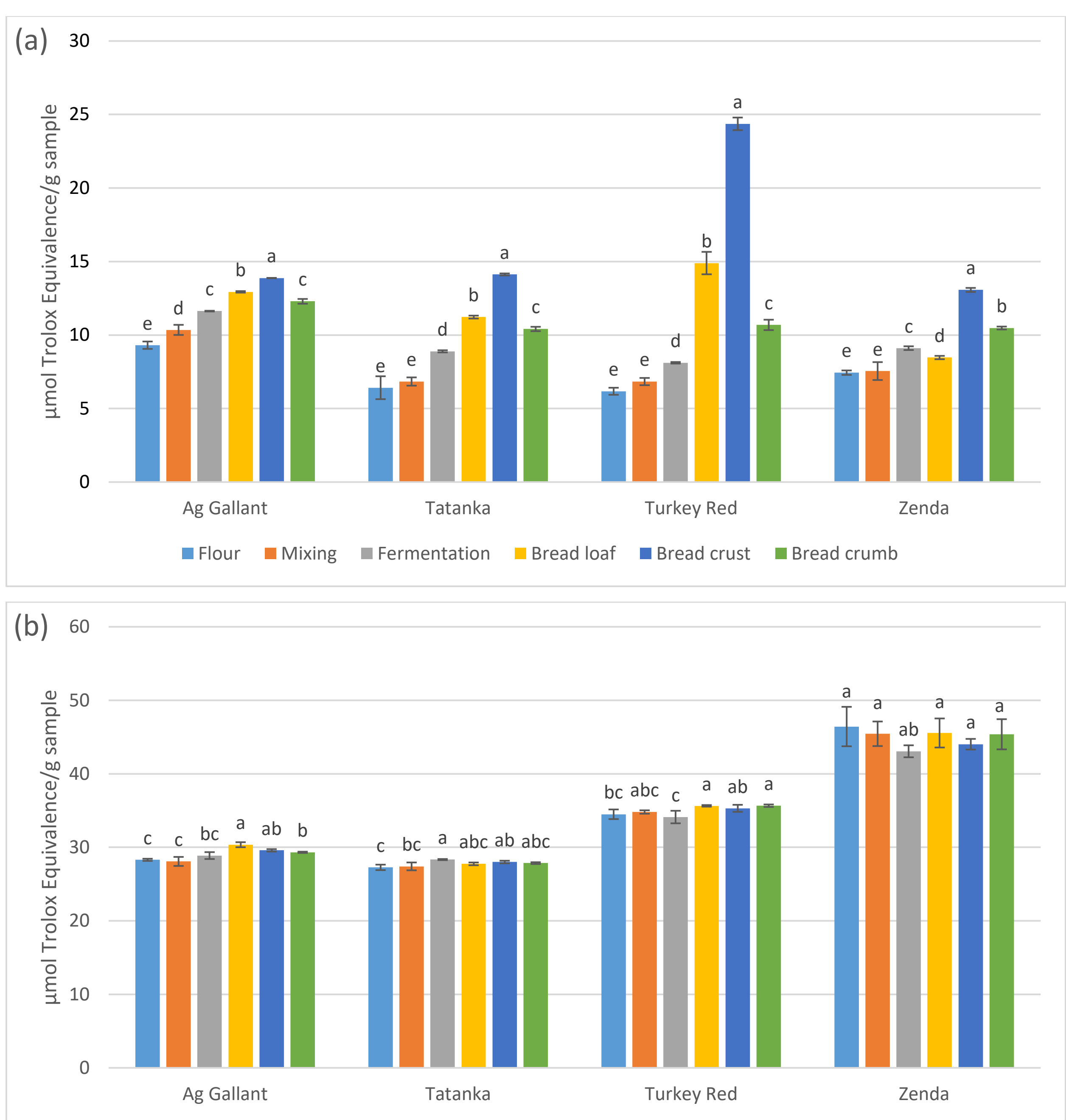
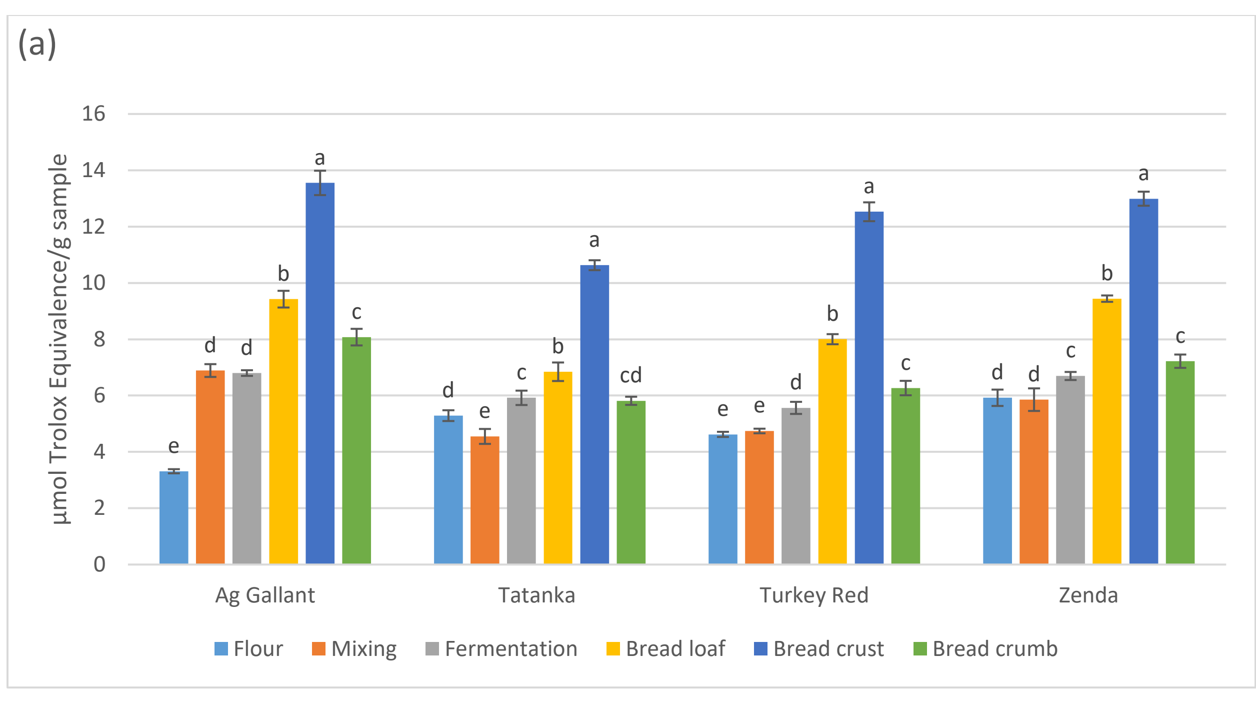


Figure 6-1 ABTS radical scavenging activity of (a)soluble and (b)insoluble phenolic compounds extracts.

Within each variety, numbers with no letter in common are considered as significantly different ($p < 0.05$).



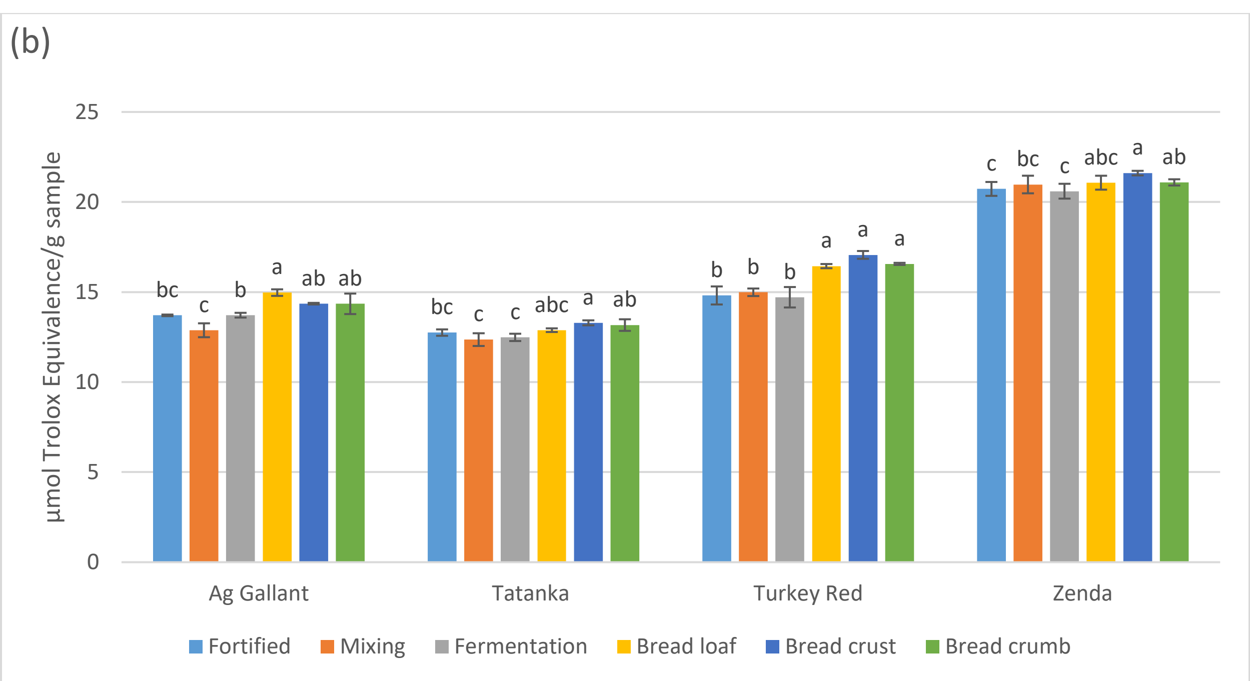


Figure 6-2 DPPH radical scavenging activity of (a)soluble and (b)insoluble phenolic compounds extracts.

Within each variety, numbers with no letter in common are considered as significantly different ($p < 0.05$).

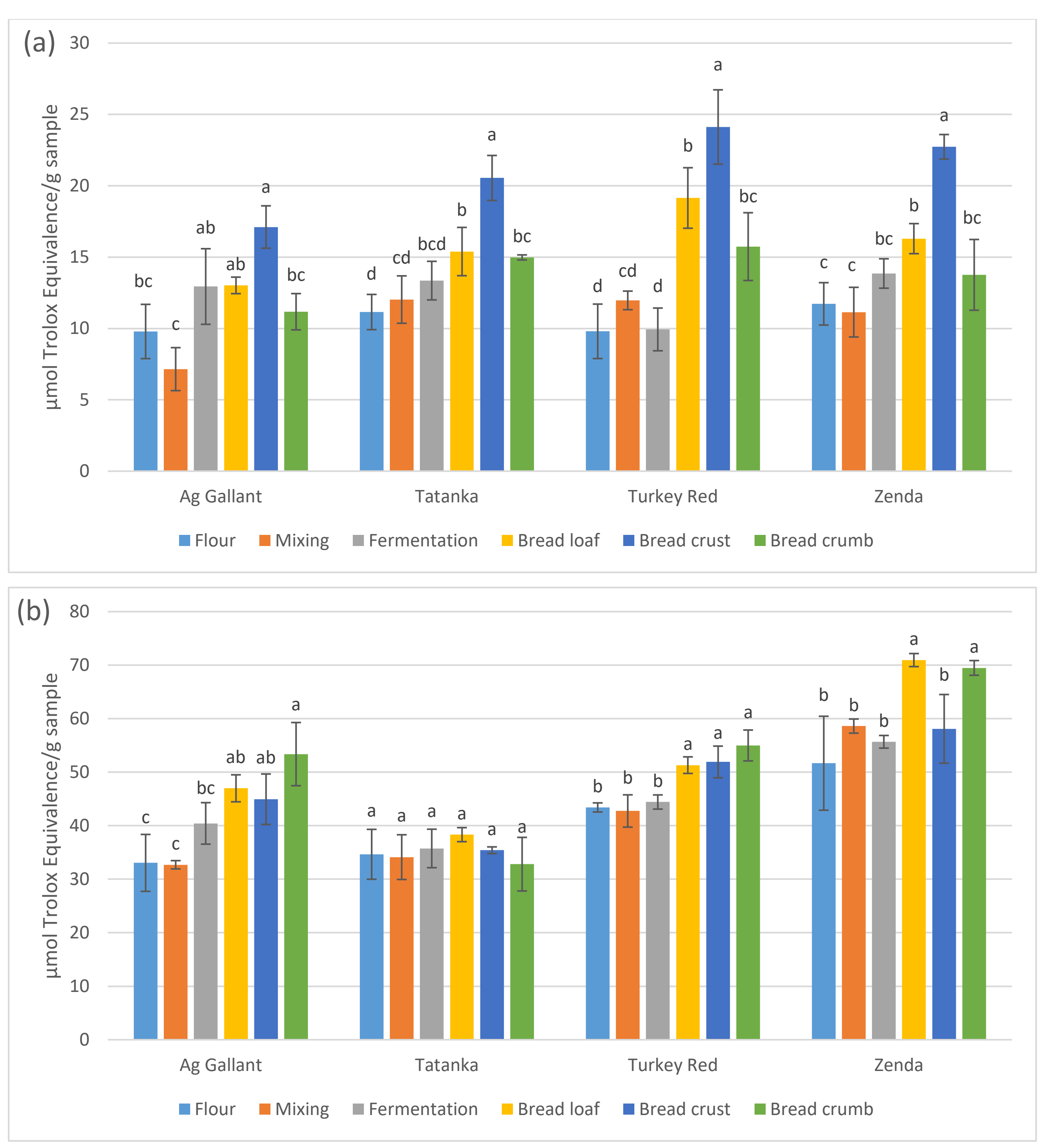


Figure 6-3 ORAC of (a)soluble and (b)insoluble phenolic compounds extracts.

Within each variety, numbers with no letter in common are considered as significantly different ($p < 0.05$).

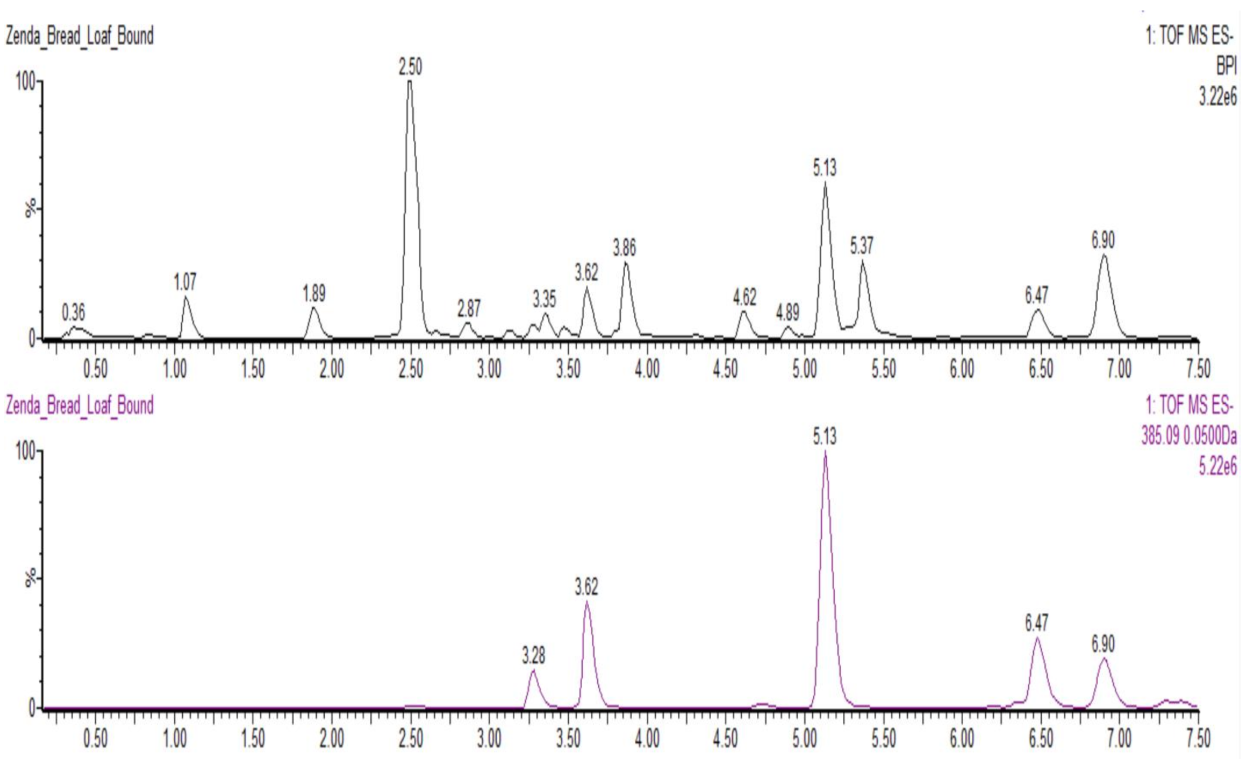


Figure 6-4 UPLC-MS spectra of a typical insoluble-bound fraction.

Upper (black): original spectrum. Lower(purple): spectrum showing peaks at m/z of 385.09 (molecular ion peak for di-ferulic acid in negative mode). Assignments of major peaks: 1.07 min: vanillic acid; 1.89 min: *p*-coumaric acid; 2.50 min: *trans*-ferulic acid, 2.87 min: cis-ferulic acid; 3.28 min: 8-8’ DFA; 3.62 min: 8-5’ DFA; 3.86 min: azelaic acid; 4.62 min: unknown; 5.13 min: 5-5’ DFA; 5.37 min: unknown; 6.47 min: 8-O-4’ DFA; 6.90 min: 8-5’ Benzofuran DFA.

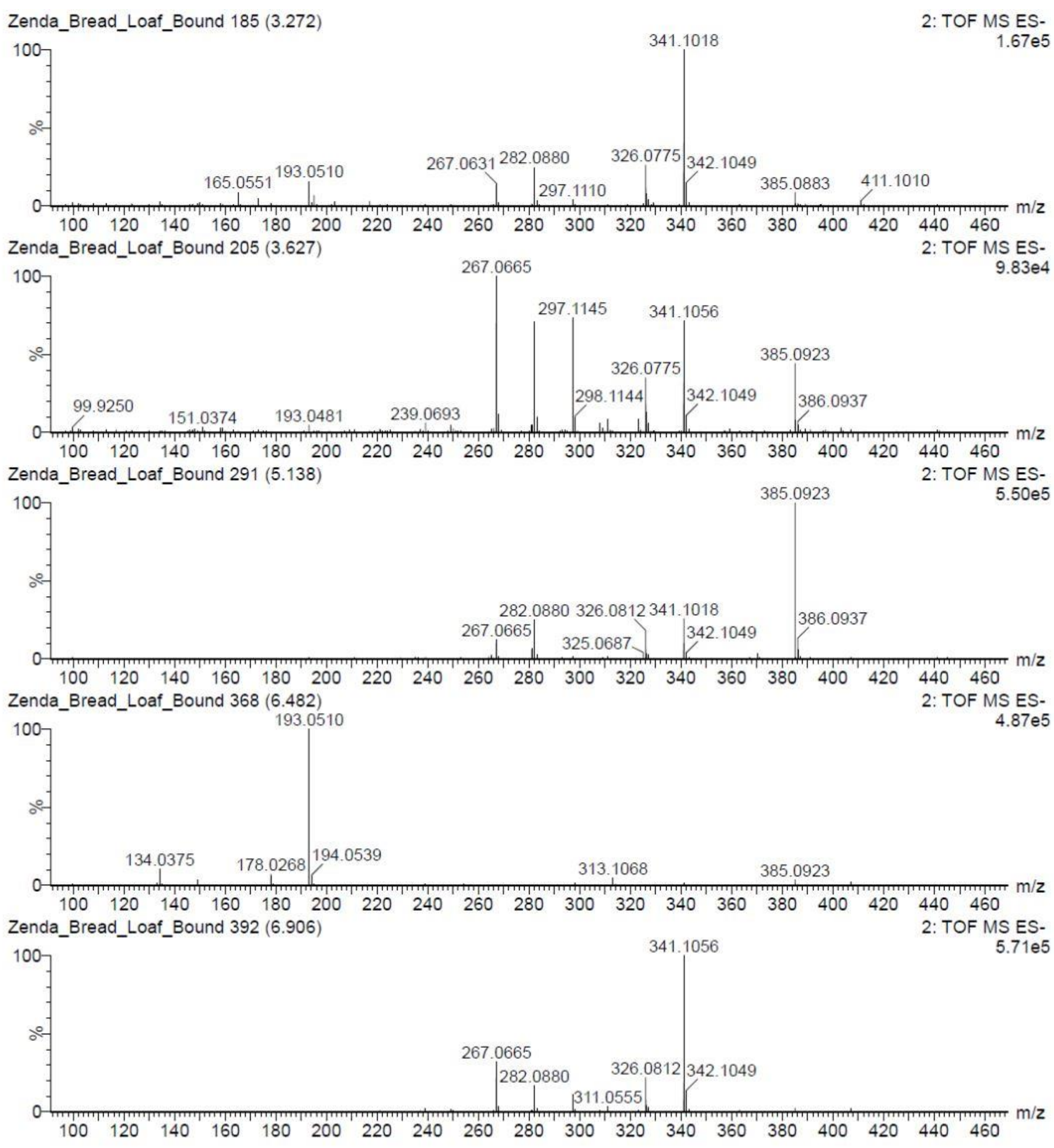


Figure 6-5 A typical example for MS^E fragmentations spectra of DFA isomers in insoluble bond fraction of bread loaf sample.

Peak assignments are as follows, 3.272 min: 8-8’ DFA; 3.627 min: 8-5’ DFA; 5.138 min: 5-5’ DFA; 6.482 min: 8-O-4’ DFA; 6.906 min: 8-5’ Benzofuran DFA.

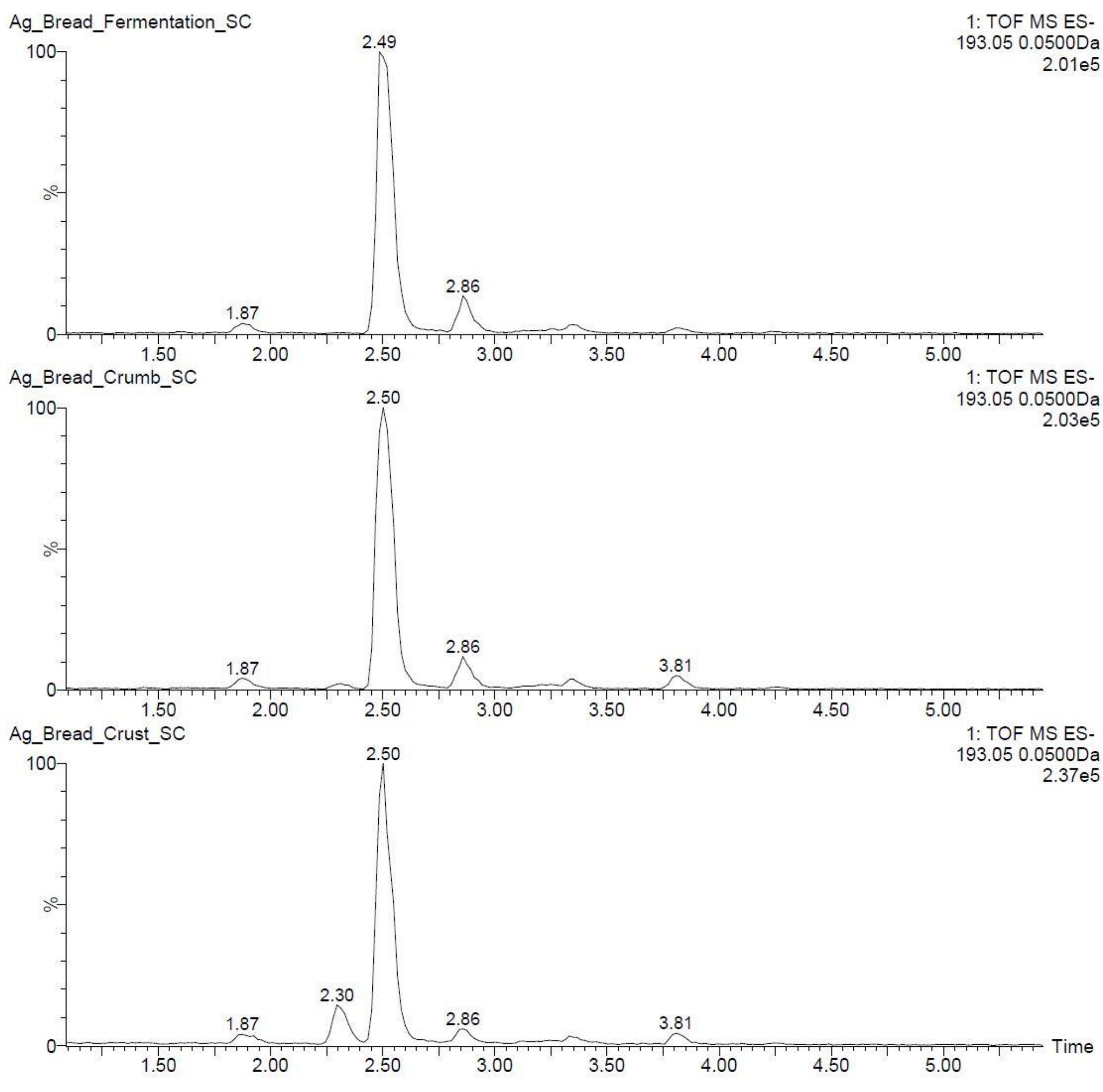


Figure 6-6 Typical MS spectra at m/z= 193.05 for the soluble-conjugated (SC) fraction of fermentation dough, bread crumb and bread crust.

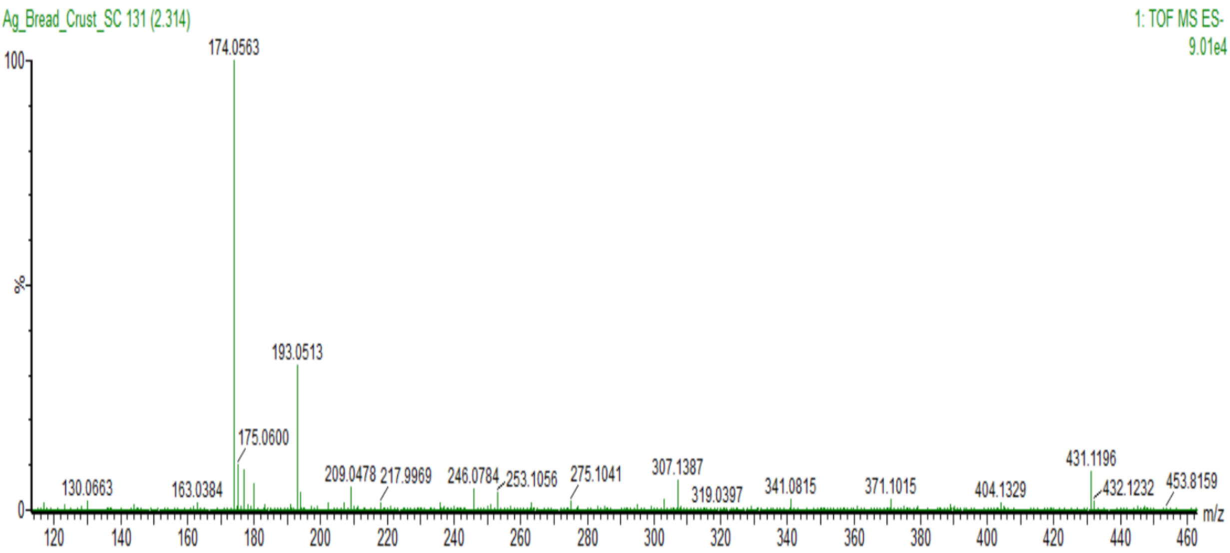


Figure 6-7 MS spectra of the peak at 2.3 minutes from bread crust that can possibly be from Maillard reaction products.

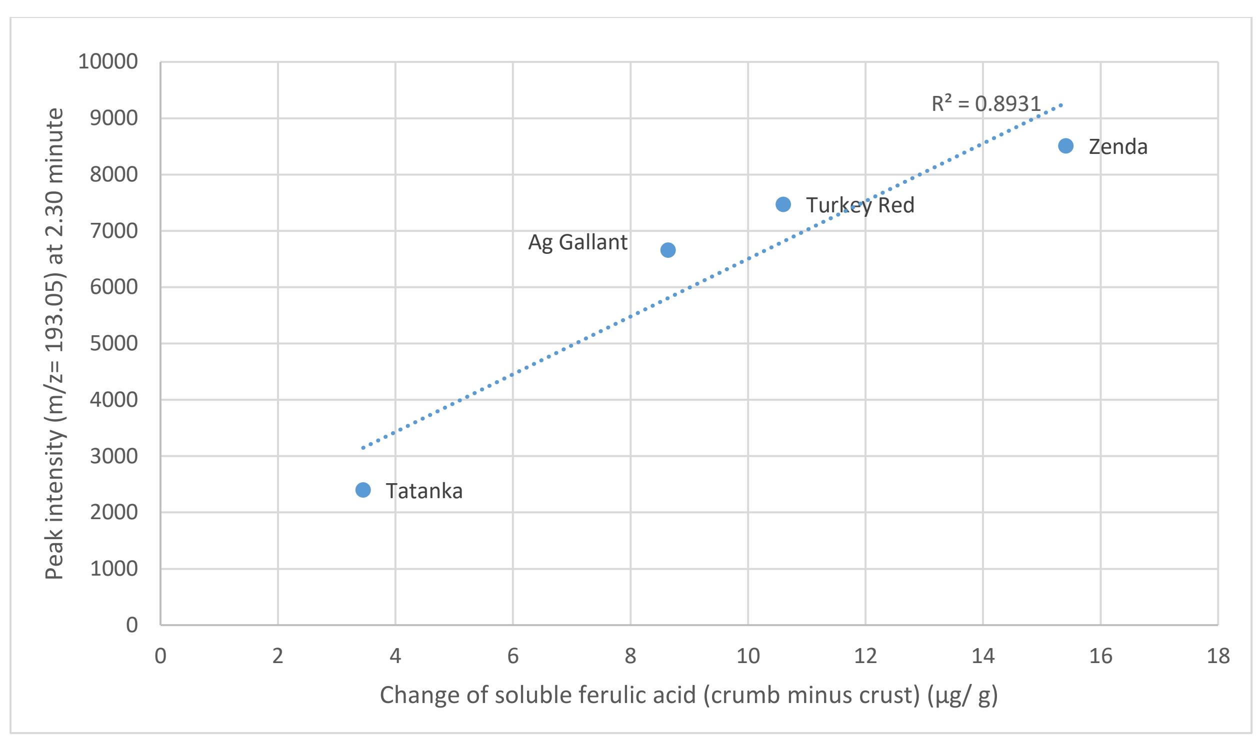


Figure 6-8 Correlation of decreased *trans*-ferulic acid concentration of bread crust (compared to bread crumb) and increased peak intensity (m/z= 193.05) at 2.30 minute that may be assigned to Maillard reaction products.

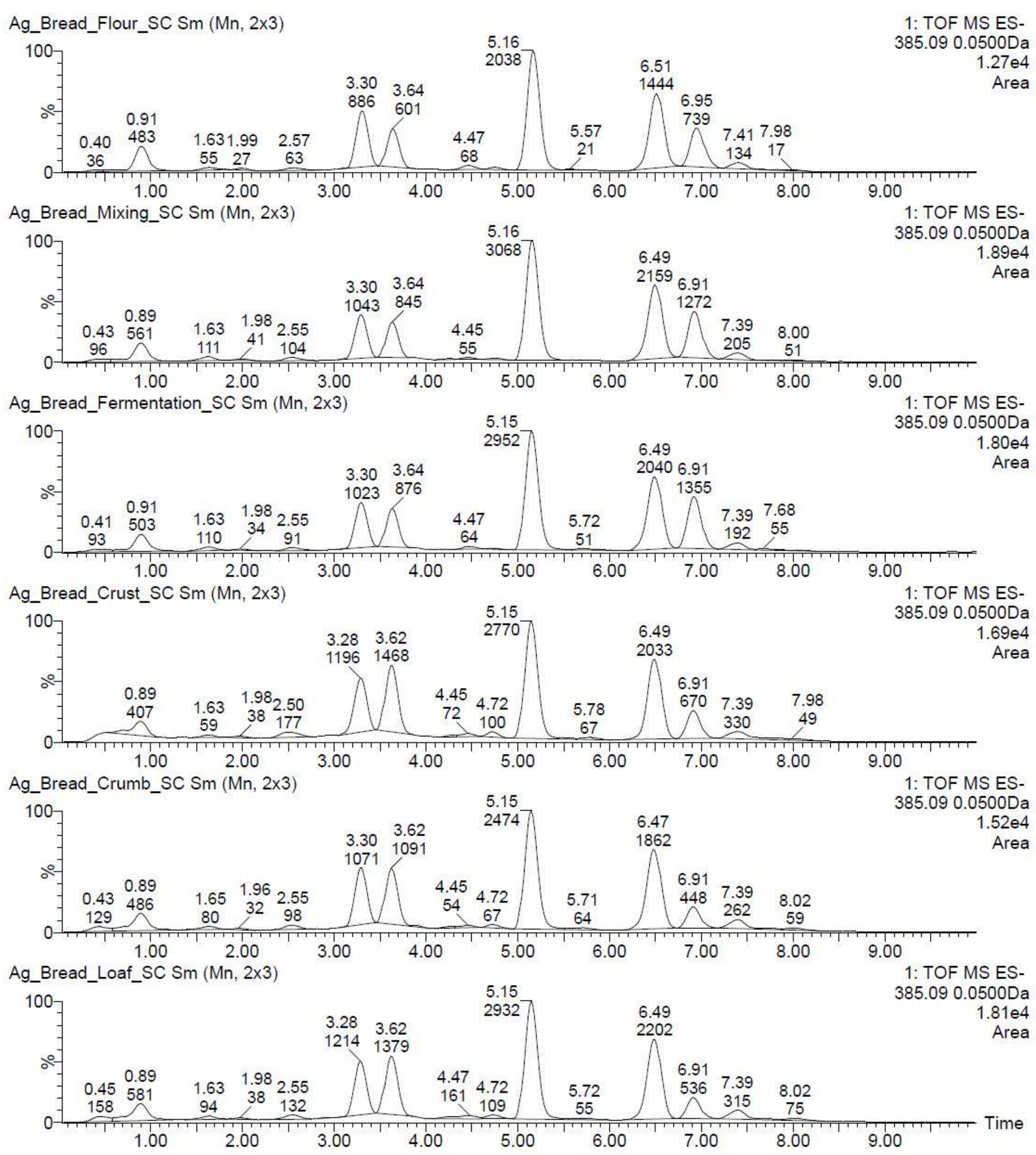


Figure 6-9 Changes of content of soluble-conjugated (SC) DFA isomers of Ag Gallant during the bread-making steps. Changes of relative peak areas (at m/z=385.09) reflect the change of absolute amount of DFA isomers that are not quantified due to lack of standard.

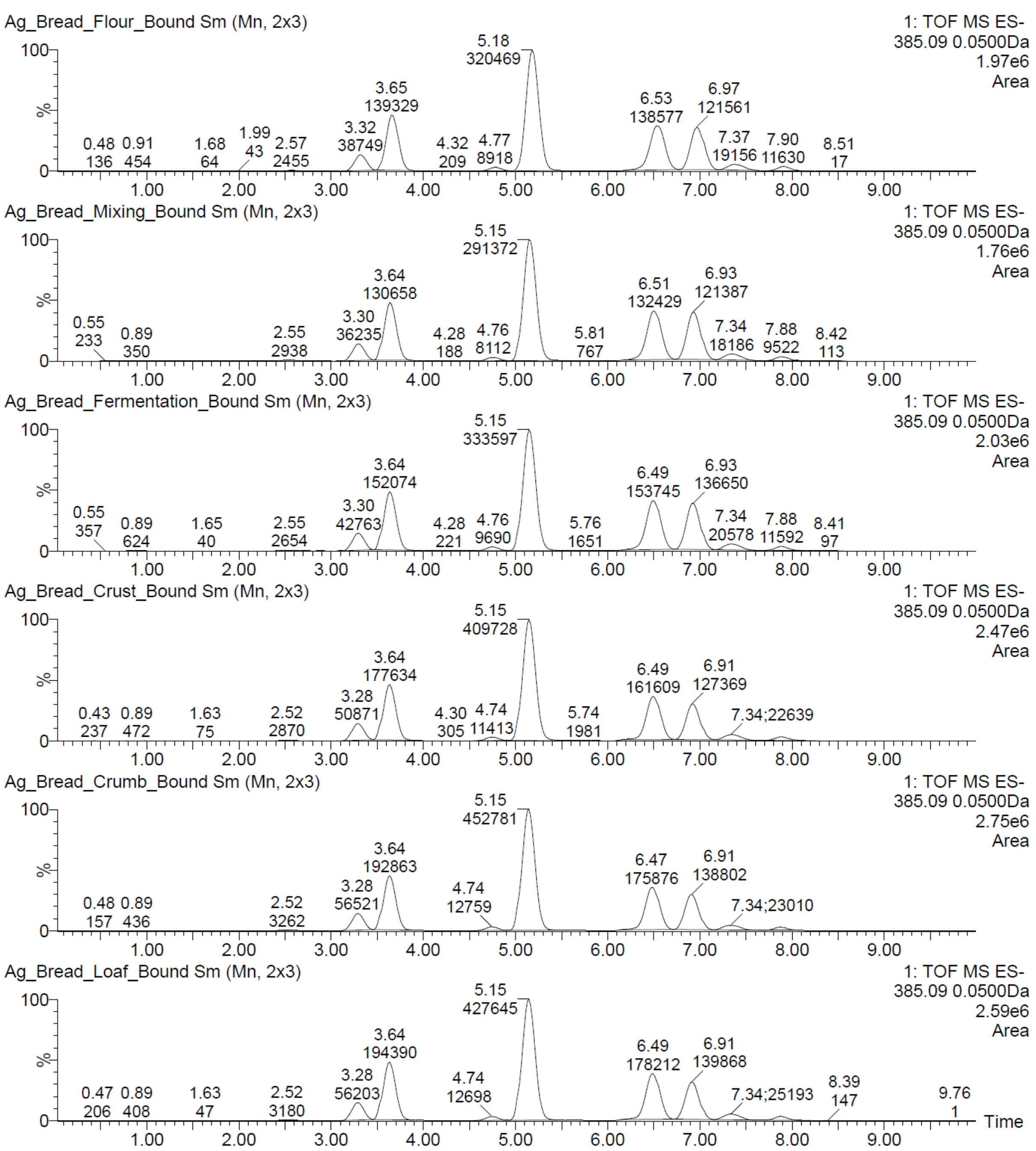


Figure 6-10 Changes of content of bound DFA isomers of Ag Gallant during the bread-making steps. Changes of relative peak areas (at m/z=385.09) reflect the change of absolute amount of DFA isomers that are not quantified due to lack of standard.

Chapter 7 - Potential bio- accessibility of phenolic acids in whole wheat products during in vitro gastrointestinal digestion and probiotic fermentation*

Abstract

Health benefits of whole wheat products are partially attributed by their unique phenolic compounds. This study investigated effect of simulated gastrointestinal digestion and probiotic fermentation on releasing of phenolic acids from whole wheat foods (bread, cookie, and pasta). Kinetics results showed that more phenolic acids were released within the first hour of gastric and intestinal digestions compared to the prolonged digestion. *Lactobacillus rhamnosus* GG, a common probiotic strain, released additional phenolic acids from the digestive residues during fermentation. Simulated digestion released more soluble *trans*-ferulic acid than chemical extraction in breads (17.69 to 102.71 µg/g), cookie (15.81 to 54.43 µg/g), and pasta (4.88 to 28.39 µg/g). Phenolic acid composition of whole wheat products appeared to be better estimated by digestion methods than the chemical extraction method. The unique insoluble-bound nature and fermentability of wheat phenolic acids may lead to a mechanistic understanding of whole grain consumption for potential colorectal cancer prevention.

* This chapter has been under review for publication. Tian, W., Hu, R., Chen, G., Zhang, Y., Wang, W., Li, Y., Potential bioaccessibility of phenolic acids in whole wheat products during in vitro gastrointestinal digestion and probiotic fermentation.

Key words: wheat phenolic acids; simulated digestion; bioaccessibility; probiotic; colon fermentation

7.1. Introduction

Consumption of whole grain products may reduce risks of chronic diseases including colorectal cancer (Okarter & Liu, 2010). The health benefits of whole grains including whole wheats can partially be attributed by their phytochemicals, especially phenolic compounds (Borneo & Edel León, 2012). Wheat phenolics usually exist in soluble-free, soluble-conjugated, and insoluble-bound forms. Insoluble-bound phenolics are of low bioaccessibility during the gastrointestinal digestion (Liu, 2007; Saulnier et al., 2007). Among of which *trans*-ferulic acid is the most abundant phenolic acid in wheat. Phenolic profiles and antioxidant activity of whole wheat have been extensively reported and reviewed (Liu et al., 2020; Zhu & Sang, 2017). However, in most previous studies, soluble phenolics are extracted using organic solvents, and insoluble-bound phenolics are hydrolyzed using concentrated NaOH solution (e.g., 2 M) (Adom et al., 2003; Moore et al., 2005). These extraction conditions are different from human digestive system. Therefore, the release and solubilization of phenolics from food matrix by human digestive systems need to be further investigated, as only those soluble phenolics can be potentially bio-accessible (Anson et al., 2009).

Some previous studies have reported the effects of simulated digestion and colon fermentation on bioaccessibility of whole grain phenolics (Adebo & Gabriela Medina-Meza, 2020; Gong et al., 2019; Zaupa et al., 2014). However, raw whole grain flours instead of the final food products were used for upper gastrointestinal (GI) digestions in most of those studies, although the flours cannot be consumed directly by humans. Our recent study found that the bread-making process affected phenolic profiles of whole wheat flours significantly (Tian et al., 2021). Therefore, whole wheat products, instead of the flours, are more suitable starting materials to understand the

release of phenolics during GI digestion. Furthermore, there are some studies on digestion of whole bread enriched with other polyphenols such as green coffee beans (Świeca et al., 2018) and quinoa leaves (Świeca et al., 2014). However, phenolics from an external source may interfere with the interpretation of phenolics released from food matrix. Another common drawback of the reported digestion studies is a lack of digestion kinetic information, as most previous studies were focused on the end points of gastric and intestinal phases. Therefore, further studies are needed to gain a more comprehensive understanding on the release of wheat phenolics from food matrix in the human digestive system.

Bread, cookie, and pasta are among the most common whole wheat foods. The objective of this study is to gain an advanced understanding on release of phenolic acids from these common whole wheat products and their bioaccessibility in human digestive system. More specifically, the objectives are to: 1) determine changes of potential bioaccessibility of phenolic acids through digestion; 2) obtain kinetic information on the release of phenolic acids from food matrix; and 3) understand the effect of probiotic fermentation on bioaccessibility of phenolic acids in whole wheat products.

7.2. Materials and methods

7.2.1. Chemicals, enzymes, and probiotics

Analytical standards (gallic acid,4-hydroxybenzoic acid, vanillic acid, syringic acid, *para*-coumarci acid, *trans*-ferulic acid, and sinapic acid), Folin- Ciocalteu reagent, α-amylase from human saliva (A1031), bile bovine (B3883), and pancreatin from porcine pancreas (P7545) were purchased from Millipore Sigma (St. Louis, MO, USA). Rabbit gastric extract (RGE) containing

pepsin (> 500 U/ mg) and gastric lipase (> 15 U/ mg) were purchased from Lipolytech (Marseille Cedex 09, France). *Lactobacillus rhamnosus* GG (LGG) was provided by Chr. Hansen A/S (Hørsholm, Denmark). LC-MS grade solvents and other general chemicals were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

7.2.2. Preparation of whole wheat food products

Whole wheat bread flour was milled in our lab according to AACC Approved Method 26-50.01. Whole pastry flour (for cookies) and whole semolina flour (for pasta) were purchased from a grocery store. Whole wheat bread and cookie were prepared following AACC Approved Method 10-10.03 and 10-50.03, respectively. Whole wheat pasta was prepared and cooked as described in our previous study (Chen & Li, 2019). Briefly, the pasta formulation contained whole semolina flour (100 g), deionized (DI) water (40 mL), and NaCl (2 g). The ingredients were mixed for 8 min in a pin mixer to develop a crumby dough. After resting for 30 min, pasta sheets were prepared using a Model 150 pasta maker (Imperia, Italy) by passing the dough through four decreasing roll gaps (2.0, 1.6, 1.2, and 0.8 mm, respectively). The whole wheat products (bread, cookie, and cooked pasta) were lyophilized, finely ground, and kept in a -20 °C freezer until further uses.

7.2.3. *In vitro* upper gastrointestinal digestion

Simulated *in vitro* digestion was performed according to the INFOGEST 2.0 digestion method (Brodkorb et al., 2019) with some minor modifications. Simulated saliva fluids (SSF), simulated gastric fluids (SGF), and simulated intestinal fluids (SIF) were prepared following exactly the INFOGEST 2.0 method and warmed to 37 °C before digestion experiments. Lyophilized sample

(1 g) was mixed with 5 mL SSF containing 12.0 mg α-amylase and 0.15mL $CaCl_2$ (0.4 M) for 2 min at 37 °C water bath, simulating the oral phase. The mixture was then acidified immediately with 0.4 mL HCl (1 M) to pH 2, and 5 mL SGF containing 16.0 mg RGE was added. The duration of simulated gastric digestion was up to 3 hours. After the gastric digestion, the sample was neutralized to pH 7 and then added with 5 mL SIF containing 20 mg pancreatin and 20 mg bile bovine. The simulated intestinal digestion was also lasted up to 3 hours. To gain insights into kinetic information during the digestion process, a total of 6 parallel samples were prepared for each whole grain food type, starting from the oral phase. One of the simulations was terminated at the first hour of the gastric digestion and labelled at Gastric-1, and the simulation terminated after the first hour of intestinal digestion was labelled as Intestinal-1. Similarly, Gastric-2, Gastric -3, Intestinal-2, and Intestinal-3 samples were obtained. Heat-shock treatment was performed to inactivate the enzymes, and the digested mixture was centrifuged at 4500 xg for 20 min. The supernatant was used for further analysis, and the residue of I-3 sample was used for the LGG fermentation. For each whole grain product, the above series of digestion was repeated for three times.

7.2.4. Simulated colonic fermentation with LGG

Residues after 3-hour intestinal digestion (I-3 sample) was lyophilized and used for probiotic fermentation. The simulated colonic fluid (SCF) contained NaCl (137 mM), KCl (2.7 mM), Na_2HPO_4 (10 mM), and KH_2PO_4 (1.8 mM) (Yadav et al., 2013). Lyophilized digestion residue after the intestinal phase (approximately 0.2 g) was mixed with LGG (100 billion CFUs) and 15 mL SCF and LGG probiotics in a 15 mL centrifuge tube. The mixture was incubated in a water bath at 37 °C for 20 hours. Then, the mixture was centrifuged at 4500 *x*g for 30 min. The

supernatant was used for analysis of soluble phenolic acids; phenolics in the residue were extracted according to a conventional alkaline extraction method to collect insoluble-bound phenolics (Adom et al., 2003; Moore et al., 2005).

7.2.5. Analysis of phenolic acid composition

Phenolic acids in the supernatant from upper GI digestion and colonic fermentation were in both soluble-free and soluble-conjugated forms. Soluble-free phenolics were analyzed directly, and soluble-conjugated phenolics were further extracted before LC-MS analysis (Gong et al., 2019). In brief, 5 ml supernatant was added with concentrated NaOH to achieve 2 M NaOH concentraton in the solution and hydrozed for 3 hours under nitrogen protection. Then, the mixture was acidified to pH 2 and extracted with ethyl acetate for five times. The combined extraction of organic phase was evaporated to dryness and reconstituted to a final volume of 3 mL using LC-MS grade methanol. Samples were stored in a -20 °C freezer and analyzed within two days. Similarly, phenolic acid composition of the whole wheat food products without digestion and fermentation were also determined according to the conventional methods (Adom et al., 2003; Moore et al., 2005). Briefly, 1 g sample was extracted with 15 mL ethanol (80%, v/v) for 1 hour to obtain the soluble fraction. Soluble-conjugated phenolic acids were further released through hydrolysis with NaOH (2 M) for 3 hours under nitrogen followed by acidification and ethyl acetate extraction. The residue was hydrolyzed with NaOH solution (2 M) for three hours under nitrogen protection followed by acidification and ethyl acetate extraction. The phenolic extracts were analyzed using a UPLC-DAD-ESI-Q-TOF-MS/MS system from Waters Corporation (Milford, MA, USA) following the same protocol reported in our previous study (Tian et al., 2021). Phenolic acids were quantified according to external standards.

Soluble-free and soluble-conjugated phenolic acids were combined as total soluble phenolic acids.

7.2.6. Total phenolic content assay

Total phenolic content(TPC) assay was performed according to our previous study (Tian & Li, 2018). In brief, the digested supernatant (0.1 mL) was thoroughly mixed with DI water (7.9 mL) and Folin- Ciocalteu reagent (0.5 mL). After 5 min, the mixture was added with 1.5 mL Na_2CO_3 solution (20%, w/v). After resting for 2 hours, absorbance of the final mixture at 765 nm was recorded using a VWR UV1600-PC spectrophotometer (Radnor, PA, USA). The result was calculated using gallic acid as an external standard and expressed as microgram gallic acid equivalence (GAE) per gram of the original sample (mg GAE/g).

7.2.7. Oxygen radical absorbance capacity

Oxygen radical absorbance capacity (ORAC) assay was performed according our previous report (Tian et al., 2019). In brief, 50 µL diluted digestion supernatant was added with 100 µL fluorescein (6 nM) in a 96-plate microplate from Corning Inc (Corning, NY). After incubation at 37 °C for 30 min, 50 µL AAPH solution (76.5 mM) was added to the mixture. The excitation and emission wavelengths were 485 and 528 nm, respectively. The intensity of the fluorescence was recorded for 2 hours with a time-step of one minute at 37 °C using a Biotek Synergy H1 Hybrid microplate reader (Winooski, VT, USA). The net area under the curve (AUC) was calculated according to the following formula:

$AUC= 0.5+ F_1/ F_0 + F_2/ F_0 +\ldots F_i/ F_0\ldots+ F_{119}/ F_0 + 0.5*F_{120}/ F_0$, where F_i = fluorescence reading at minute i. The ORAC value of the digestion supernatant was quantified according to an

external standard curve and expressed as micromole Trolox equivalence per gram of sample (μmol TE/ g).

7.2.8. Statistical analysis

The results were presented as mean ± standard deviation (SD) from three replicates. One-way analysis of variance (AVONA) and Turkey's test were performed using SAS software, version 9.4 (Cary, NC, USA) for significant analysis ($p < 0.05$).

7.3. Results and discussions

7.3.1. Phenolic acids composition at different GI digestion stages

Phenolic acid composition of the whole wheat bread, cookie, and cooked pasta at different stages of simulated GI digestion is summarized in **Table 7-1**. For bread, the amount of soluble *tran*-ferulic acid increased significantly from 44.82 to 63.36 μg/g from the first hour of gastric digestion to the end of gastric digestion (i.e., Gastric-3). Further intestinal digestion (1 hour) significantly ($p< 0.05$) increased the soluble *tran*-ferulic acid to an amount of 85.10 μg/g. Similar trend was observed for 4-hydroxybenzoic acid, vanillic acid, and sinapic acid. Increased duration at the intestinal phase did not further release *tran*-ferulic acid but had a significantly positive ($p<0.05$) effect on sinapic acid. Concentration of sinapic acid increased from 18.93 to 26.95 μg/g during the last two hours of intestinal digestion. For cookie and pasta, average amounts of soluble *trans*-ferulic acid also increased from gastric phase to intestinal phase. Interestingly, intestinal digestion greatly released 4-hydroxybenzoic acid in all the three food matrices (i.e., bread, cookie, pasta). Comparing the end point of gastric (Gastric-3) and intestinal digestion (Intestinal-3), soluble 4-hydroxybenzoic acid increased by 2.6-fold for cookie (from 4.80 to

12.50 µg/g) and 5-fold for pasta (1.89 to 9.46 µg/g). Intestinal digestion also greatly enhanced amount of sinapic acid and *cis*-ferulic acid in the pasta sample. Sinapic acid, instead of *trans*-ferulic acid, was the most abundant phenolic acid in pasta digesta. We previously reported that sinapic acid may be more abundant than *trans*-ferulic acid in the soluble fraction for some wheat varieties (Tian et al., 2019).

Generally, the digestion process apparently increased concentration of soluble phenolic acids, and therefore increased their potential bioavailability. Insoluble-bound 4-hydroxybenzoic acid and sinapic acid were found to be extremely sensitive to intestinal digestion. Most changes of phenolic acid composition occurred within the first hour of gastric and intestinal digestions. This result also suggested that digestive enzymes were the major driving force for release of phenolic acids and the contribution from solubilization in the digestive fluids was almost negligible. To our knowledge, this is the first study reporting detailed kinetic information regarding changes of phenolic acids during the digestion process.

7.3.2. Total phenolic content and antioxidant activity of digested whole grain products

Total phenolic content (TPC) and oxygen radical absorbance capacity (ORAC) are common *in vitro* assays measuring antioxidant potential of wheat and other food products. Though there are some limitations of the assays such as non-specificity, TPC and ORAC assays are still of great value, especially when the analyte contains phenolic compounds with a diverse and oligomeric nature that elude HPLC quantification (Beer et al., 2004; Granato et al., 2018). TPC and ORAC results are plotted in **Figure 7-1** and **Figure 7-2**, respectively. For bread, TPC of Gastric-2h

(1.91 mg GAE/g) was significantly ($p<0.05$) higher than that of Gastric-1h digesta (1.62 mg GAE/g). However, for cookie and pasta samples, extended digestion duration from 1 hour to 2 or 3 hours did not lead to higher TPC values. Different types of wheat (hard red winter, soft wheat, and durum wheat for bread, cookie, and pasta, respectively) and different whole food processing conditions may be responsible these differences. Besides, bread flour was lab-milled and the bran particle size was relatively larger, while cookie and pasta flours were commercially milled and the brans were of relatively smaller size. Brans with smaller particle sizes are more accessible to digestive enzymes (Brewer et al., 2014). Compared to the end point of gastric phase, intestinal digestion greatly increased TPC and ORAC values, which indicated further release of bio-active components during intestinal digestion. This observation was different from a previous study on pasta digestion showing that TPC was not further increased during the intestinal phase compared to the gastric phase (Podio et al., 2019). In that study, the mixture was homogenized at 22,000 rpm for 30 s during the simulated oral phase, which might enhance the pasta digestion in the later gastric phase. Gong et al. (2019) reported that TPC of digested cereal grains ranged from 2.89 (wheat) to 4.23 (corn) mg GAE/g. Our result was consistent with this report, with TPC of digested bread, cookie, and pasta of 3.87, 3.65, and 4.00 mg GAE/g, respectively.

As discussed in a previous study (Gong et al., 2019), TPC of digested whole wheat (2.89 mg GAE/g) was much higher than the control value (0.55 mg GAE/g for non-digested whole wheat) obtained by conventional organic extraction. The amount of soluble *trans*-ferulic acid, the major phenolic acid in whole wheat, was still less than 0.1 mg/g after digestion (**Table 7-1**). Therefore, besides phenolic acids, there must be other significant contributors to the increased TPC after

digestion. We previous reported that enzymatic hydrolysis can increase TPC and antioxidant activities of cereal proteins (Hu et al., 2020; Xu et al., 2019). Digestive enzymes may also release bio-active peptides and amino acids that significantly contributed to the TPC and antioxidant activity assays. Another study on sugars showed that though fructose and glucose did not directly react with Folin-Ciocalteu reagents, they exhibited enhanced synergistic effect with phenolic compounds and led to an overestimation of total phenolic content assay result (Magalhães et al., 2010). In summary, phenolics including phenolic acids, peptides, amino acids, and other bioactive compounds together contributed to the increased TPC and antioxidant activities of whole wheat products after digestion. *In vitro* assay methods have been widely used to understand processing effect and for quality control purpose (Granato et al., 2018), further studies are needed to investigate the physiological mechanisiums of the increased assay values.

7.3.3. Comparison of chemical and GI digestive method

Phenolic acids in whole wheat and other products are commonly determined by conventional chemical extraction method (i.e., soluble fraction was collected by organic solvent extraction, and insoluble fraction was further released from the residue of organic extraction by using 2 M NaOH) (Moore et al., 2005). Based on that method, the soluble *trans*-ferulic acid in the bread, cookie, and pasta was 17.69, 15.81 and 4.88 μg/g, respectively, and the insoluble *tran*-ferulic acid was 549.25, 254.17, and 274.40 μg/g, respectively. Most *trans*-ferulic acids (> 94%) existed in insoluble-bound fractions. This observation was in agreement with previous studies (Adom et al., 2003; Tian & Li, 2018). Phenolic acids in insoluble-bound forms are not considered as bioaccessible (Liu, 2007). For the digested samples, soluble phenolic acids were released by simulated digestions. Insoluble-bound phenolic acids were further released by conventional

NaOH hydrolysis from the digested residues. **Figure 7-3(a), 7-3(c), 7-3(e)** present phenolic acid composition determined by the conventional method for bread, cookie, and pasta, respectively, and **Figure 7-3(b), 7-3(d), 7-3(f)** show phenolics released from digestion and chemical extraction for bread, cookie and pasta, respectively. Simulated digestion significantly increased percentage of soluble *trans*-ferulic acid for bread (3.12% to 15.44%), cookie (6.00% to 17.55%), and pasta (1.78% to 8.62%). Majority of the insoluble-bound *trans*-ferulic acid can reach the colon as reported previously (Liu, 2007).

Digestion methods apparently released more 4-hydroxybenzoic acid, vanillic acid, and sinapic acid. After the simulated digestion, all sinapic acids exist in soluble form and no sinapic acid was detected in the insoluble from. The amount of sinapic acid released by digestion was 29.92, 18.93, and 30.76 μg/g for bread, cookie, and pasta, respectively. These values were much higher than sinapic acids extracted by conventional methods, which were only 18.43, 11.76, and 12.92 μg/g, respectively, for bread, cookie and pasta. Similarly, majorities of 4-hydroxybenzoic acid (68.57, 56.58, 100%) and vanillic acid (69.05, 50.80 and 32.50%) were also released by the simulated digestion for bread, cookie, and pasta. Digestion released higher amounts of 4-hydroxybenzoic acid and vanillic acid than the conventional chemical extraction method. For example, simulated digestion and fermentation released 12.64 μg/g of the soluble vanillic acid from the cookie, while the conventional method can only release 4.50 μg/g soluble and 5.24 μg/g insoluble vanillic acid. Furthermore, additional 12.24 μg/gof vanillic acid was extracted by alkaline hydrolysis from the digestion residue. It was possible that vanillic acid was naturally embedded within the fiber-protein network. Under conventional extraction conditions, these complexes are more resistant to organic solvents extraction as well as NaOH treatment;

therefore, they cannot be extracted by the conventional method. Digestive enzymes weakened the network so that more vanillic acid can be released from the digestive residue. In summary, these results suggested that conventional alkaline hydrolysis may not release all phenolic acids from insoluble-bound forms in the food matrix. A recent study also claimed that phenolic profile of whole grains can be better estimated by simulated digestion than conventional chemical extraction (Danesi et al., 2020). It will be necessary to develop enzyme-assisted extraction protocols to maximize the extraction potential of phenolic acids from wheat flours and products in future study.

7.3.4. Release of phenolic acids by probiotic fermentation

Though the upper GI digestion released some wheat phenolic acids into soluble forms, most *trans*-ferulic acids can still reach the colon in the insoluble-bound forms (Liu, 2007). In this study, we performed probiotic fermentation with LGG, a strain that has been extensively studied and commercialized (Weichselbaum, 2009). After probiotic fermentation, the residues were extracted using conventional NaOH hydrolysis to fully release insoluble-bound phenolics. Phenolic acid composition from fermentation is summarized in **Table 7-2(a)**, and the composition of phenolic acids extracted from the fermentation residue is summarized in **Table 7-2(b)**. LGG fermentation further released *trans*-ferulic acid and some other phenolic acids from the GI digestion residues. The concentration of *trans*-ferulic acid released during bread, cookie and pasta fermentation were 13.70, 9.96, and 7.44 μg/g, respectively. Additional 4-hydroxybenzoic acid, vanillic acid, and syringic acids were also detected in the extracts of fermented bread and cookie samples, but not in the pasta samle. We also found that if no LGG was added, mixing digestion residues within the SCF for 20 hours did not release any detectable

phenolic acids from the food matrix. Therefore, it can be confirmed that LGG fermentation released insoluble phenolic acids, although majority of the phenolic acids still remained in the fermentation residues (Table 2b). Hole et al. (2012) also reported that *Lactobacillus johnsonii* LA1, a probiotic strain commercially used in fermented milks, released 56.50 out of 674.48 (total) µg/g and 53.76 out of 271.32 (total) µg/g *trans*-ferulic acid from barley and oat flour, respectively. Though the study (Hole et al., 2012)) may overestimate probiotics' ability to release phenolic acids in human colon because no upper gastrointestinal digestion was performed before fermentation, both the literature and our study confirmed that colon microbiota can release insoluble phenolic acids. Differently, Anson et al. (2009) reported that colon fermentation with human feces did not increase soluble phenolic acids but increase phenolic metabolisms. In our study, we only tested the performance of one single bacteria strain, but this system can be used for the screening of probiotics strains. Food industry advocating health benefits of added probiotics can possibly use this simple fermentation model for faster selection of probiotics strains especially for fermenter milks with added whole grains.

7.4. Conclusions

Gastrointestinal digestion released some bound phenolic acids into soluble forms in whole wheat products. Compared to phenolic acid composition determined by conventional chemical extraction methods, wheat phenolic acids released by simulated digestion exhibited higher proportions of soluble fractions and higher total amounts of 4-hydroxybenzoic acid, vanillic acid, and sinapic acid. Majority of the insoluble *trans*-ferulic acids can survive upper GI digestion and reach the colon. Our probiotic fermentation experiments confirmed that colonic microbiota can release certain amount of phenolic acids including *trans*-ferulic acid from the digested residues.

Released phenolic antioxidant in the colon may partially explain the contribution of whole grain consumption to the colon health. Future studies with true animal or human feces seem warranted for better understanding of the potential bioaccessibility of wheat phenolic acids in the colon.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 7-1 Changes of phenolic acid composition during simulated upper GI digestion.

	4-HA (μg/g)	VA (μg/g)	SyrA (μg/g)	pCA (μg/g)	tFA (μg/g)	SipA (μg/g)	cFA (μg/g)
Bread							
Gastric-1	7.48±0.07e	12.29±0.36ab	4.42±0.47cd	1.09±0.38b	44.82±4.34c	9.27±0.46c	2.07±0.03ab
Gastric-2	8.81±0.14d	10.25±0.50b	7.30±0.61ab	1.39±0.02ab	50.71±2.50c	10.67±0.61c	2.89±0.05ab
Gastric-3	10.35±0.19c	12.79±0.49ab	8.22±0.57a	1.70±0.05ab	63.36±2.21b	11.97±0.28c	4.00±0.12a
Intestinal-1	16.10±0.46b	26.12±1.74a	3.71±0.43d	1.31±0.58ab	85.10±4.96a	18.93±0.85b	1.65±1.98b
Intestinal-2	15.29±0.36b	22.79±10.38ab	3.36±1.00d	1.77±0.04ab	85.72±4.21a	22.78±3.80ab	3.18±0.28ab
Intestinal-3	17.03±0.39a	21.66±7.54ab	5.99±0.17bc	1.96±0.02a	89.01±3.02a	26.95±4.75a	3.49±0.57ab
Cookie							
Gastric-1	4.84±0.12b	5.98±1.47b	2.24±0.31b	1.66±0.02a	36.91±1.60ab	12.83±0.46a	3.35±0.17a
Gastric-2	4.88±0.07b	7.61±0.93ab	4.07±1.05a	1.75±0.25a	35.87±3.67b	15.90±4.32a	0.76±1.20b
Gastric-3	4.80±0.19b	10.25±2.53a	3.26±0.58ab	1.75±0.14a	37.37±3.81ab	16.48±4.56a	1.70±0.47b
Intestinal-1	12.17±0.37a	8.80±0.30ab	3.36±0.46ab	1.97±0.08a	42.59±1.06ab	17.05±0.43a	1.57±0.03b
Intestinal-2	12.42±0.28a	8.58±0.14ab	3.48±0.37ab	1.96±0.03a	43.14±3.38ab	17.54±0.70a	1.59±0.05b
Intestinal-3	12.50±0.12a	8.71±0.61ab	2.55±0.67ab	2.00±0.07a	44.47±3.22a	18.93±0.32a	1.68±0.00b
Pasta							
Gastric-1	2.01±0.07b	3.21±0.05bc	1.27±0.08e	1.09±0.38b	15.93±1.20b	15.44±2.11b	5.31±0.80b
Gastric-2	1.97±0.07b	3.43±0.05bc	1.56±0.03d	1.35±0.02b	15.82±1.66b	16.75±0.56b	5.80±0.21b
Gastric-3	1.89±0.07b	2.93±0.06c	1.66±0.02d	1.37±0.02b	15.92±2.02b	16.99±0.02b	5.90±0.09b
Intestinal-1	9.22±1.06a	4.47±0.55ab	3.70±0.02b	1.91±0.00a	22.38±0.46a	30.81±0.68a	11.10±0.26a
Intestinal-2	8.89±0.44a	4.97±1.04a	4.31±0.72a	1.90±0.02a	21.70±2.34a	30.85±0.80a	11.12±0.30a
Intestinal-3	9.46±0.25a	3.90±0.20abc	3.08±0.05c	1.90±0.02a	20.95±1.85a	30.77±0.93a	11.09±0.35

Results are expressed as mean ± std, n=3. Means in a box without a common letter differ ($p < 0.05$). 4-HA: 4-hydroxybenzoic acid; VA: vanillic acid; SyrA: syringic acid; pCA: *para*-coumaric acid; tFA: *trans*-ferulic acid; SipA: sinapic acid; cFA: *cis*-ferulic acid.

Table 7-2 (a) Additional phenolic acids released by LGG fermentation (b) Phenolic acids release from fermentation residues by conventional alkaline hydrolysis.

	4-HA (μg/g)	VA (μg/g)	SyrA (μg/g)	pCA (μg/g)	tFA (μg/g)	SipA (μg/g)	cFA (μg/g)
(a) fermentation							
bread	1.73±0.28b	2.14±0.00b	0.62±0.15b	nd	13.70±0.30a	2.97±1.17a	nd
cookie	4.44±0.14a	3.93±0.16a	1.41±0.15a	0.65±0.01a	9.96±0.20b	nd	nd
pasta	nd	nd	nd	nd	7.44±0.09	nd	nd
(b) phenolics extracted from fermentation residues by conventional methods							
bread	8.60±0.12b	10.67±0.06b	2.23±0.07a	10.41±0.11a	487.44±3.39a	nd	23.51±0.51a
cookie	13.00±0.05a	12.24±0.11a	0.86±0.02b	4.65±0.22c	208.91±0.89c	nd	9.06±0.11b
pasta	nd	8.12±1.66c	0.69±0.02c	6.48±0.06b	221.93±2.61b	nd	nd

Results are expressed as mean ± std, n=3. Means in a box without a common letter differ ($p < 0.05$). 4-HA: 4-hydroxybenzoic acid; VA: vanillic acid; SyrA: syringic acid; pCA: *para*-coumaric acid; tFA: *trans*-ferulic acid; SipA: sinapic acid; cFA: *cis*-ferulic acid.

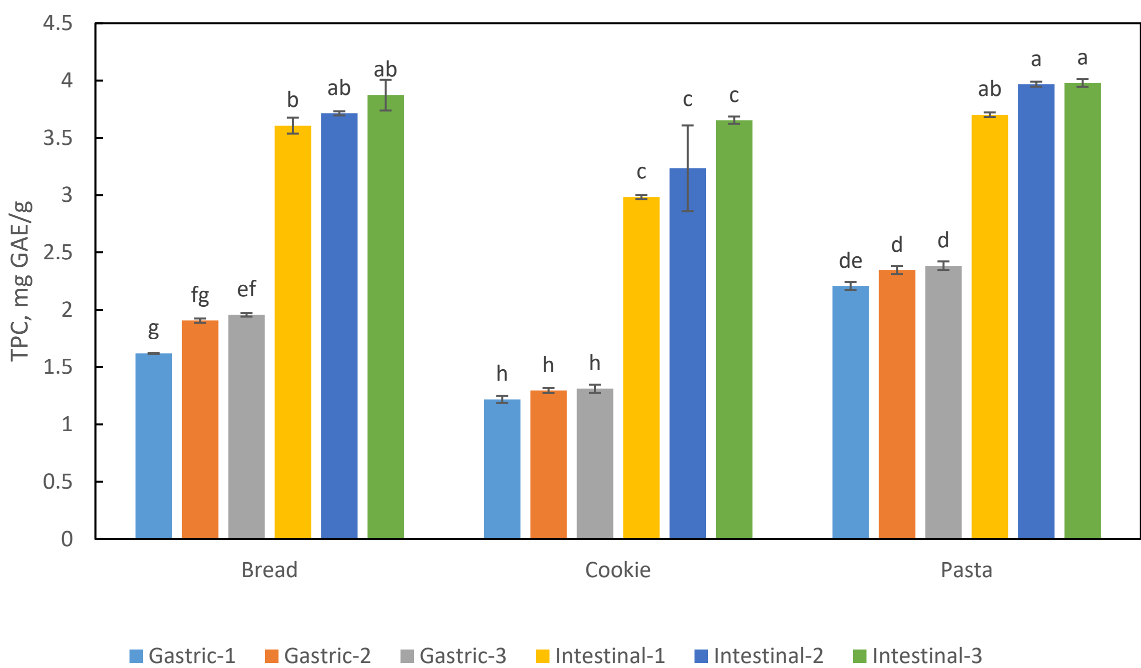

Figure 7-1 Changes of total phenolic content (TPC) of whole grain products during simulated GI digestion.

Results are expressed as mean ± std, n=3. Bars without a common letter differ ($p < 0.05$).

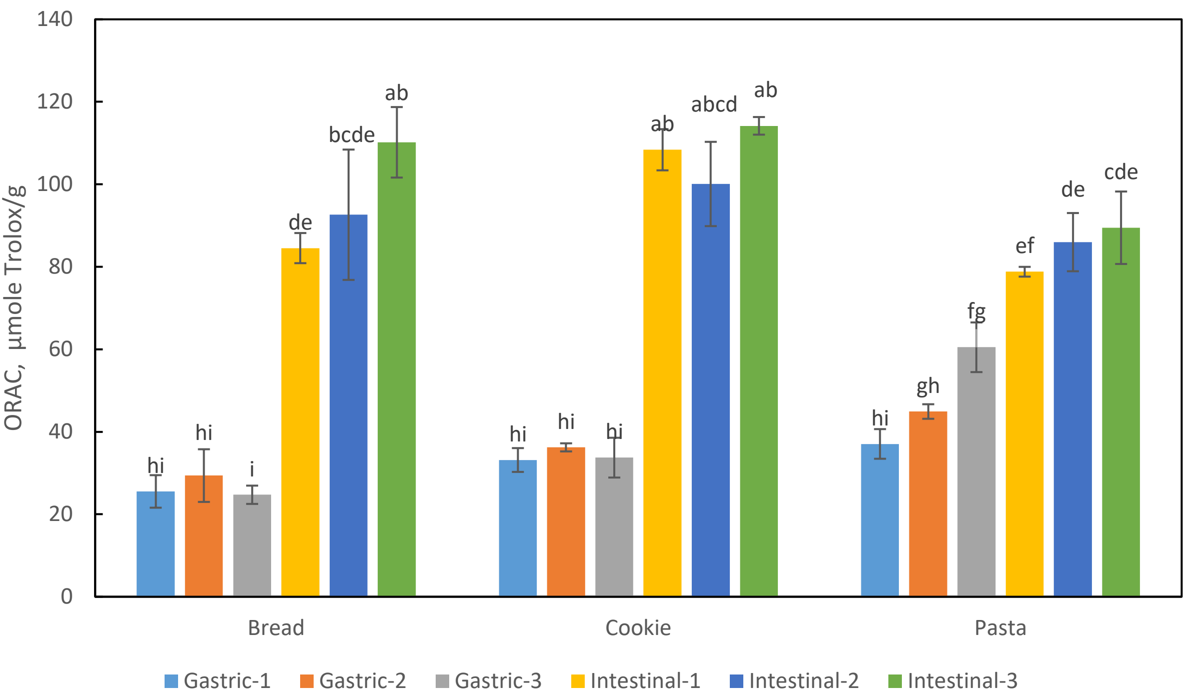

Figure 7-2 Changes of oxygen radical absorbance capacity (ORAC) values of whole grain products during simulated GI digestion.

Results are expressed as mean ± std, n=3. Bars without a common letter differ ($p < 0.05$).

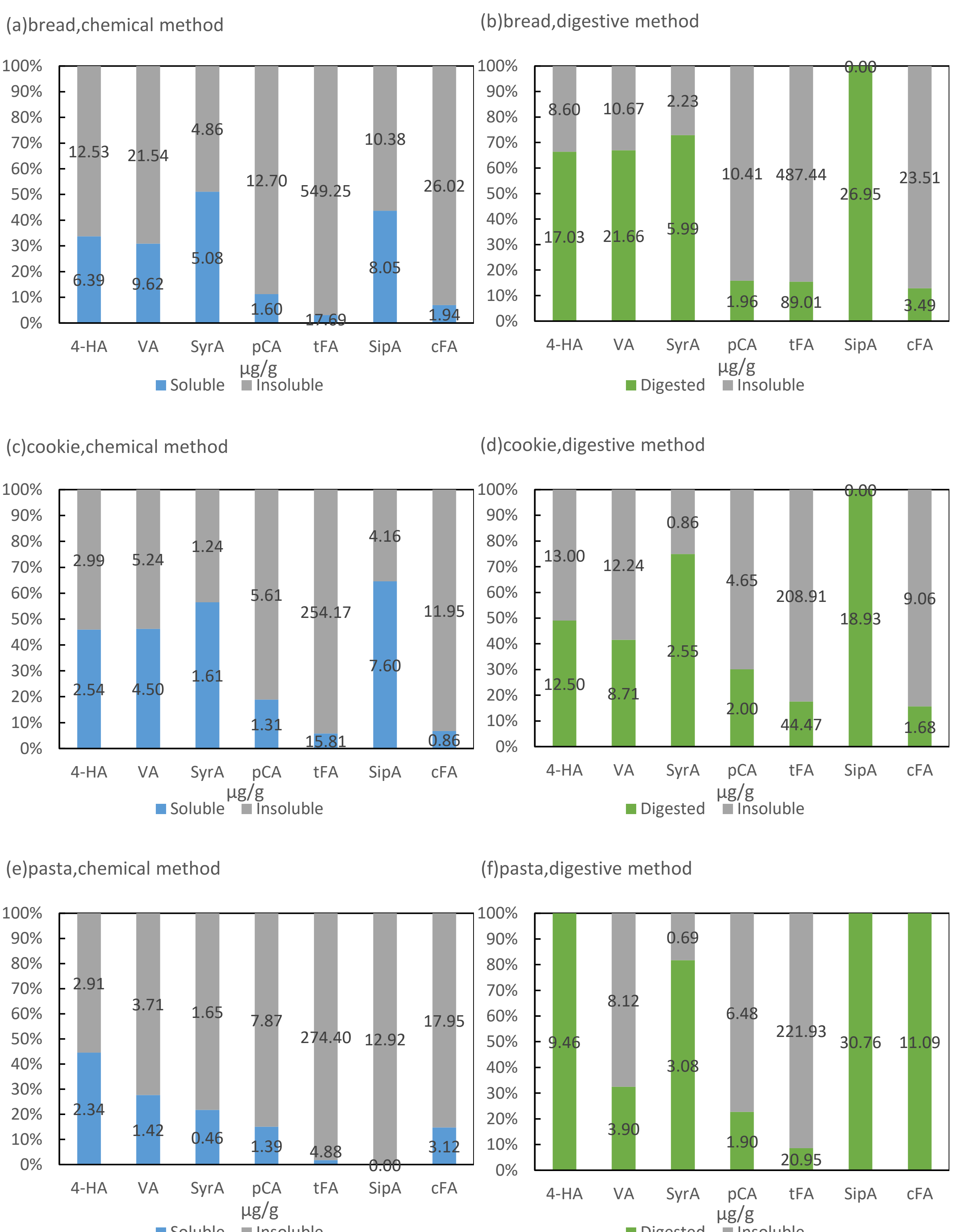
(a)bread,chemical method
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
12.53
21.54
4.86
12.70
549.25
10.38
26.02
6.39
9.62
5.08
1.60
17.69
8.05
1.94
4-HA
VA
SyrA
pCA
tFA
SipA
cFA
µg/g
Soluble
Insoluble
(b)bread,digestive method
0.00
8.60
10.67
2.23
10.41
487.44
26.95
23.51
17.03
21.66
5.99
1.96
89.01
3.49
µg/g
Digested
Insoluble
(c)cookie,chemical method
2.99
5.24
1.24
5.61
254.17
4.16
11.95
2.54
4.50
1.61
1.31
15.81
7.60
0.86
µg/g
Soluble
Insoluble
(d)cookie,digestive method
0.00
13.00
12.24
0.86
4.65
208.91
18.93
9.06
12.50
8.71
2.55
2.00
44.47
1.68
µg/g
Digested
Insoluble
(e)pasta,chemical method
2.91
3.71
1.65
7.87
274.40
12.92
17.95
2.34
1.42
0.46
1.39
4.88
0.00
3.12
µg/g
Soluble
Insoluble
(f)pasta,digestive method
0.69
8.12
6.48
221.93
9.46
3.08
30.76
11.09
3.90
1.90
20.95
µg/g
Digested
Insoluble

Figure 7-3 Phenolic acid profile of whole wheat products determined by conventional and digestion methods.

Results are expressed as mean ± std, n=3. 4-HA: 4-hydroxybenzoic acid; VA: vanillic acid; SyrA: syringic acid; pCA: *para*-coumaric acid; tFA: *trans*-ferulic acid; SipA: sinapic acid; cFA: *cis*-ferulic acid.

Chapter 8 - Rapid determination of total phenolic content and ferulic acid in whole wheat using UV-Vis spectrophotometry*

Abstract

Phenolic compounds in wheat products have the benefits of maintaining health and reducing risks of some chronic diseases and cancers. Wheat breeders and producers are becoming interested in producing wheats with higher amount of bioactive phenolics, in addition to the conventional targets of yield, disease and pest resistance, milling and baking quality, etc. Generally, total phenolic content (TPC) is determined using Folin- Ciocalteu reagent, and ferulic acid needs to be quantified through chromatography method. These procedures are time-consuming and labor-intensive. In this study, we developed partial least squares (PLS) models for rapid and accurate determination of both TPC and ferulic acid of whole wheat extract based on data collected using microplate reader UV-VIS spectroscopy. The spectra ranged from 240 to 360nm were used for model development. A total of 60 samples were used as calibration set, and 20 samples were used as external validation set. For the TPC model, the R^2-calibration is 0.89, and R^2-validation is 0.89. For ferulic acid model, R^2-calibration is 0.82, and R^2-validation is 0.85. Compared with the traditional analytical methods, our approach is more convenient and rapid and remains comparable accuracy. Development of the new models will significantly speed up the screening and selection processes of wheats with promising phenolic content when large number of samples need to be analyzed, such as during wheat breeding.

Key Words: UV-Visible model; wheat phenolics; ferulic acid; PLS regressions

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8.1. Introduction

Consumption of whole wheat products has been associated with reduced risk of chronic diseases(Liu, 2007). Many health benefits are attributed to the dietary fibers as well as wheat phytochemicals(Liu, 2004). Majority of the wheat phytochemicals are phenolic compounds. Driven by consumers' desire for health-beneficial and functional foods, phytochemical content and composition are becoming a niche but important attribute in evaluating wheat quality (Moore et al., 2005). To assess the potential health benefits of whole wheat, many experimental assays have been developed, such as total phenolic content (TPC), total flavonoid content (TFC), DPPH radical scavenging activity, ABTS radical scavenging activity, oxygen radical absorbance capacity, and others (Pisoschi & Negulescu, 2012). Among these assays, TPC is the most common assay (López-Alarcón & Denicola, 2013). Various wheat phenolic acids such as *p*-hydroxybenzoic acid, vanillic acid, syringic acid, caffeic acid, p-coumaric acid, ferulic acid and sinapic acid have been identified and quantified by HPLC method (Kim et al., 2006). Ferulic acid, a natural antioxidant, is the most abundant phenolic acid in wheat (Tian & Li, 2018).

However, the currently available methods in quantifying wheat phytochemicals, such as TPC assay and HPLC protocols, are relatively time-consuming and labor-intensive. For example, a single HPLC run for identification and quantification of phenolic acids usually takes up to or even more than 20 minutes (Moore et al., 2005; Okarter et al., 2010). TPC assay involves several hazardous chemicals and generates a large amount of chemical waste (Singleton & Rossi, 1965). These drawbacks significantly limit and slow down the analysis of wheat phenolics when large numbers of samples need to be preliminarily screened and tested, such as in wheat breeding

programs to identify wheat traits and lines for potential health benefits. Therefore, it is imperative to develop rapid and effective methods for the determination of TPC and phenolic acids in wheat. Meanwhile, for the typical assays for wheat phenolic analysis, phenolic extracts are always prepared as soluble-free, soluble-conjugated, and insoluble-bound fractions, though it is widely agreed that the insoluble-bound form accounts for over 90 percent of total wheat phenolics (Adom et al., 2003; Moore et al., 2005; Tian & Li, 2018). For large wheat breeding programs, a simplified extraction protocol that allows the extraction of total wheat phenolics is preferred.

Regression model method based on spectral data is a potential alternative to traditional analytical and wet chemistry methods in the determination of some constituents in food materials. Reference data and regression algorithms are crucial for model development. The reference data are generally collected using primary assays depending on the specific component being analyzed. Partial least square (PLS) regression and principal component regressions (PCR) are common regression methods for model development. Cross validations, residue predictive deviation (RPD) and external validation data set have been used for model evaluation. Near-infrared spectroscopy (NIR) is a non-destructive method and requires fewer sample preparation steps. NIR models have been developed to determine the content of protein, moisture, fat, sugars, hemicellulose and cellulose in various materials (Jia Chen et al., 2017; Jing Chen et al., 2013; K. Zhang et al., 2017) and commercially widely used. A few recent studies reported using NIR model for prediction of total phenolic contents of apple (Schmutzler & Huck, 2016), rice grains (Zhang et al., 2008) onion and shallot (Lu et al., 2011) and gluten-free grains including buckwheat, millet and oat (Wiedemair et al., 2019) However, NIR analysis normally requires

larger amount of samples, which may not be always available, especially at the early stage of wheat breeding. On the other side, UV-Vis models have been found to be promising for the determination of less concentrated bioactive compounds. Based on UV-Vis spectra information, (Naczk et al., 2002) developed a multivariate model for total phenolic acid determination of canola and rapeseed and achieved a R^2-calibration of 0.93 for the model. Luthria (2012) reported a simplified UV model for the estimation of phenolic acids and antioxidant capacity of eggplant. Martelo-Vidal & Vázquez (2014) developed PCR calibration models for syringic acid and oenin determination in red wine, with R^2-calibration of 0.84 and 0.80, respectively, and RPD values of 2.08 and 1.72, respectively. However, there has been no study on the prediction and determination of total phenolics and phenolic acids in wheat and other cereals based on regression models.

In this study, we employed a simplified protocol for the extraction of total wheat phenolics (Loreto et al., 2018). The objective was to develop a multivariate regression model based on UV-Vis spectra of the extract for rapid, simplified, and accurate determination of total phenolic content and amount of ferulic acid in whole wheat. To the best of our knowledge, this is the first reported regression models that can rapidly determine wheat TPC and ferulic acid. Screening efficiency focused on wheat phenolics in breeding or survey programs will be significantly improved by the realization of this technique, such as in the HEALTHGRAIN program(Li et al., 2008; Ward et al., 2008). Development of similar types of models are also likely to advance the research on phenolic compounds and other phytochemicals in other cereal grains such as sorghum, corn and oat.

8.2. Materials and methods

8.2.1. Wheat samples and chemicals

A total of 80 hard red winter wheat samples of various varieties were collected from the Kansas Wheat Performance Test program in 2018 and 2019. Among these, 60 samples were used as calibration set, and 20 samples were used as external prediction (validation) set. Folin- Ciocalteu reagent and standards of phenolic acids were purchased Sigma-Aldridge (St. Louis, MO, USA). Solvents and other general chemicals were purchased from Fisher Scientific (Waltham, MA, USA).

8.2.2. Extraction of total wheat phenolics

Wheat kernels were milled to fine flour using a coffee grinder, and total wheat phenolics were extracted according to a previous method (Loreto et al., 2018). One gram of wheat flour was mixed with 10 mL 80% ethanol for 20 minutes, and then 5 mL 6M NaOH was added to the slurry. The mixing was continued for 3 hours in dark in a shaker under N_2 protection. Then, the sample was acidified with 6M HCl to pH= 2. The final slurry was centrifuged at 4000 xg for 30 minutes to collect the supernatant, which was then extracted 3 times with ethyl acetate. The pooled organic phase was evaporated to dryness using a rotary evaporator and re-dissolved in 3 mL HLPC-grade methanol. The product was filtered through a 0.45 µm filter and stored at -20 °C for further analysis.

8.2.3. Total phenolic content determination using Folin- Ciocalteu assay

Total phenolic content was determined according to the traditional method (Singleton & Rossi, 1965) with minor modifications in our lab (Tian & Li, 2018). In brief, 0.1 mL phenolic

extraction was mixed with 7.9 mL deionized (DI) water and then with 0.5 mL Folin-Ciocalteu reagent. After 5 minutes, 1.5 mL 20% Na_2CO_3 solution was added. The final mixture was allowed to stand for 2 hours for color development, and the absorbance at 765 nm was recorded using VWR UV1600-PC spectrophotometer (Radnor, PA, USA). Gallic acid was used as a standard to establish the calibration curve for the quantification.

8.2.4. Phenolic acid analysis using HPLC

Phenolic acids were separated using an Agilent 1100 HPLC system (Santa Clara, CA, USA) as we previously described (Tian et al., 2019). In brief, mobile phase A was HPLC-grade water containing 0.1% trifluoroacetic acid (TFA), and mobile phase B was acetonitrile. Percentage of mobile phase B was kept at 10% from 0-10 minutes, increased linearly from 10% to 20% from 10 to 17 minutes, and kept at 20% from 17 to 22 minutes. Pure phenolic acids were used as external standards for identification and quantification.

8.2.5. Collection of UV-Vis spectra

The phenolic extract was diluted 80 times with HPLC-grade methanol. The diluted sample (200 μL) was added to UV-transparent 96-well microplate (Corning, NY, USA). Methanol (200 μL) was used as the reference. The BioTek Synergy H1 Hybrid (Winooski, VT, USA) microplate reader was set to scan the sample to collect UV-Vis absorption spectra through 240 to 600 nm with an interval of 5 nm. For each wheat sample, spectrum was collected in four replicates, and averaged spectrum was used for model development.

8.2.6. Model development

The Unscrambler 11 by CAMO software (Oslo, Norway) was used for model development. For all the spectral data, segment ranged from 240 to 360 nm was used for model development, because the sample has minimal absorbance beyond 360 nm. Preliminary principal component analysis (PCA) was performed to detect sample outliers. Calibration models for TPC and phenolic acids were established using principal component regressions (PCR) and PLS regression. Cross validation and analysis of residual variance were used for the development of calibration model to reduce potential overfitting issues. External validation (i.e., prediction) test was performed to further confirm the performance of the calibration models.

8.3. Results and discussions

8.3.1. Determination of regression model

Figure 8-1 shows raw UV spectra of all the wheat samples. Spectra beyond 360 nm showed extremely low absorbance, and therefore not used for model development. Ultraviolet absorbance at 280 nm is considered as a general characteristic of most phenolic compounds (Aleixandre-Tudo et al., 2017). Ferulic acid has the strongest absorbance at 320 nm (Pan et al., 2002). Although spectra pre-treatments such as smoothing, derivatives, and standard normal variate (SNV) were performed in many modeling studies before model development (Martelo-Vidal & Vázquez, 2014; Tahir et al., 2017), our preliminary tests suggested that the original spectra in our study showed best overall performance. Therefore, the original spectra without further pre-treatment were used for model development. Potential outliers were checked by principal component analysis (**Figure 8-2**), and the Hotelling's T test suggested that there was no significant outlier spectrum in the tested samples. Comparison between PCR and PLS regression

models suggested that the PLS regressions had better performance than the principal component regression (PCR) models (**Table 8-1**), since the PLS models had higher values of R^2-calibration.

8.3.2. Determination of optimal number of latent variables

For the PLS regression models, it is important to select optimal number of latent variables (i.e., factors), which are not directly observed. They are rather inferred through a mathematical model from other directly observed variables. If too many latent variables are used, the calibration may be overfitted, so the obtained model becomes overly dependent on the calibration data set and results in poor predictions for unknown samples. In our study, the number of latent variables was decided according to two aspects. Firstly, cross-validation is a useful tool preventing against overfitting. Although a larger R^2-calibration value suggests better correlation between predicted and reference values, a model that has closer R^2-calibration and R^2-cross validation values is less likely to have overfitting issues, and therefore is more suitable to predict unknown samples (Li et al., 2013). Secondly, it is generally agreed that models with more factors are inherently less robust (Faber & Rajkó, 2007). If one additional factor was added, and the increase of R^2 was not significant, then the current number of latent variables can be considered optimal. The Unscrambler software uses residual variance to quantify this process, which refers to the variance that cannot be explained by current regression model. If the decrease of residual variance is less than 6% (Unscrambler software default value) with increased factor number, then the current number of factors is generally optimal.

Table 8-2 shows changes of R^2 and residual variance along the number of factors used in TPC and ferulic acid models. For TPC, when the factor number increased from two to three, increased

residual variance was observed, which was not desirable. In the meantime, the two-factor model had closer R^2-calibration and R^2-cross validation values. Therefore, a model with two factors was selected for TPC. For ferulic acid, a model with four factors was selected because further increasing the number of factors did not lead to significant decrease of residual variance. Also, further increased factors resulted in more discrepancy between R^2-calibration and R^2-cross validation, which may suggest overfitting issue. Predictability of the calibration models with proposed optimal latent variable numbers were further confirmed using an external validation set.

8.3.3. Calibration and validation of regression model for TPC

Table 8-3 summarizes descriptive statistics of TPC and parameters of PLS calibration models. For the model development, R^2-calibration, root mean square error of calibration (RMSEC), root mean square error of cross validation (RMSECV), and residual prediction deviation (RPD) were used to evaluate model robustness. For the TPC model, R^2-calibration was 0.89, which suggested high correlation between reference values and predicted values of the calibration set. Cross validation was performed to reduce potential overfitting issue. Residual prediction deviation is defined as the ratio between standard deviation (SD) and RMSECV. A higher RPD value normally suggests better predictability of the calibration model. RPD was commonly used in various models, but the threshold was not specified. Generally, a model with RPD higher than 2.0 is considered more desirable, and a model with a RPD between 1.5 and 2.0 is considered fair (Cozzolino et al., 2011; Martelo-Vidal & Vázquez, 2014; Shen et al., 2010). The RPDs of our TPC and ferulic acid models are 3.0 and 2.2, respectively, which implied that the models are likely to provide excellent prediction for unknown samples.

Figure 8-3(a) shows details of the two-factor calibration model of TPC. The first factor explained 88% of the change of Y-variable (**Figure 8-4**). Regression coefficients plot of Factor-1 is shown in **Figure 8-5**. To further confirm the TPC predictability of the calibration model, additional 20 samples were used as the validation set. Prediction results are provided in **Figure 8-3(b)**. Predicted values based on the calibration model matched very well with the reference values, with a large R^2-validation of 0.89, which was also close to the R^2-calibration. As described above, we decided selecting two-factor model based on residual variance analysis. For this external validation set, when three-factor or four-factor model was used, the R^2-validation became 0.87 and 0.81, respectively. The independent validation set analysis further supported the effectiveness of the two-factor calibration model.

8.3.4. Calibration and validation of regression model for phenolic acids

Similarly, calibration models for the determination of ferulic acid and other phenolic acids were developed, and the model parameters are summarized in **Table 8-3**. **Figure 8-6(a)** shows the overview of the calibration model with four factors. Values of R^2-calibration and R^2-cross validation were 0.82 and 0.78, respectively. For the external validation set, R^2-validation was 0.85 (**Figure 8-6(b)**). For most data points, reference values agreed very well with the predicted values. When five-factor or six-factor model was used, R^2-validation became 0.83 and 0.82, respectively. This analysis also confirms our selection of number of factors for ferulic acid model. Therefore, total amount of ferulic acid in wheat extract can be rapidly predicted using the calibration model.

We also attempted to develop calibration models for other phenolic acids. The values of R^2-calibration for syringic acid, *p*-coumaric acid, and sinapic acid were 0.47, 0.57, and 0.30, respectively (**Table 8-3**), which indicated that the current models cannot achieve accurate predictions for these phenolic acids. This was probably because the amount of these phenolic acids in the extract (average 20.5-82.3 μg/g) was extremely lower compared to ferulic acid (average 664 μg/g). Therefore, specific spectral information of these phenolic acids was weak, which does not allow a robust model development using the current methods.

8.4. Conclusions

In summary, we developed effective PLS models for the prediction of both total phenolic content and amount of ferulic acid in whole wheat extract based on UV-Vis spectra. Our models provide highly accurate predictions especially for the total phenolic content. Compared with conventional wet chemistry methods, the new models are more efficient and less labor-intensive. Wheat breeding programs as well as other projects focusing on the health benefits of whole wheat that require fast screening and selection of wheat lines with significant phenolic compounds will be greatly benefitted by this new model.

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Conflict of interest

The authors declare no conflict of interest.

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Figures and tables

Table 8-1 Comparison between PLS and PCR method.

	R^2-calibration (TPC)	R^2-cross validation (TPC)	R^2-calibration (ferulic acid)	R^2-cross validation (ferulic acid)
PLS	0.89	0.89	0.82	0.78
PCR	0.87	0.86	0.79	0.74

PLS: partial least squares PCR: principal component regression

Table 8-2 Changes of PLS regression performance along the number of factors used.

Number of factors	R^2-calibration	R^2-cross validation	Residual variance (RV)	Percentage of RV decrease (%)
Total phenolic content				
0	0	0	56605	-
1	0.88	0.88	7011	87.6
2	**0.89**	**0.89**	**6403**	**8.7**
3	0.90	0.88	6709	+4.8 (increase)
4	0.92	0.90	5906	-
5	0.92	0.89	6152	-
6	0.92	0.89	6156	-
7	0.93	0.85	8482	-
Ferulic acid				
0	0	0	4242	-
1	0.71	0.70	1286	69.7
2	0.74	0.72	1195	7.1
3	0.79	0.75	1047	12.4
4	**0.82**	**0.78**	**918**	**12.3**
5	0.83	0.79	902	1.7
6	0.85	0.80	867	3.9
7	0.87	0.81	810	6.6

Table 8-3 Summary of descriptive statistics and PLS calibration models.

	mean	SD	range	R^2-calibration	R^2-cross validation	RMSEC	RESECV	RPD
TPC (µg GAE/g)	1984	243	1403-2451	0.89	0.89	76.2	80.0	3.0
Ferulic acid (µg/g)	664	67	511-809	0.82	0.78	27.1	30.3	2.2
Syringic Acid(µg/g)	55.8	32.7	17.8-166.3	0.47	0.48	25.6	28.6	1.1
p-Coumaric acid (µg/g)	20.5	4.4	20.5-32.7	0.57	0.53	2.9	3.1	1.4
Sinapic acid (µg/g)	82.3	40.9	5.6-154.4	0.30	0.28	31.6	30.0	1.4

GAE: gallic acid equivalence, SD: standard deviation, RMSEC: root mean square error of calibration, RMSECV: root mean square error of cross validation, RPD: residual prediction deviation, which equals to SD/RMSECV. Reference TPC values were determined using Folin-Ciocalteu assay. Phenolic acids were separated and quantified by HPLC method.

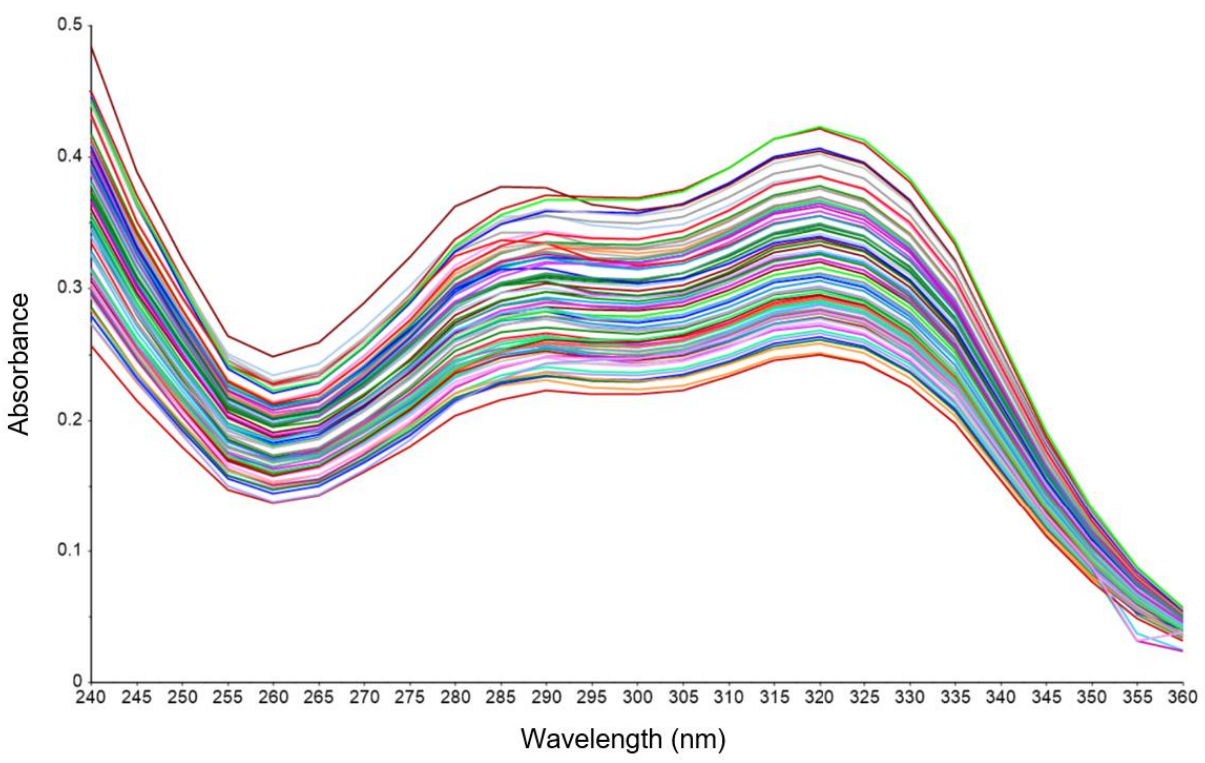

Figure 8-1 Raw UV-Vis spectra of whole flour extract.

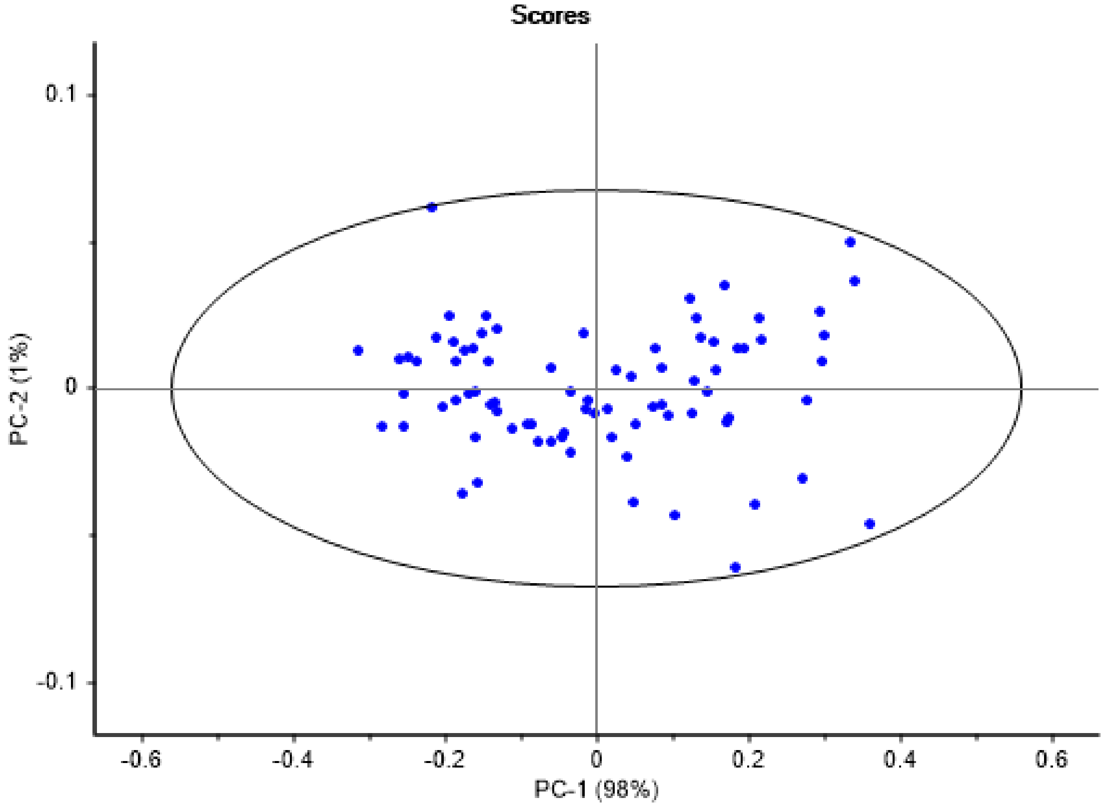

Figure 8-2 Score plot of two principal components from raw spectra

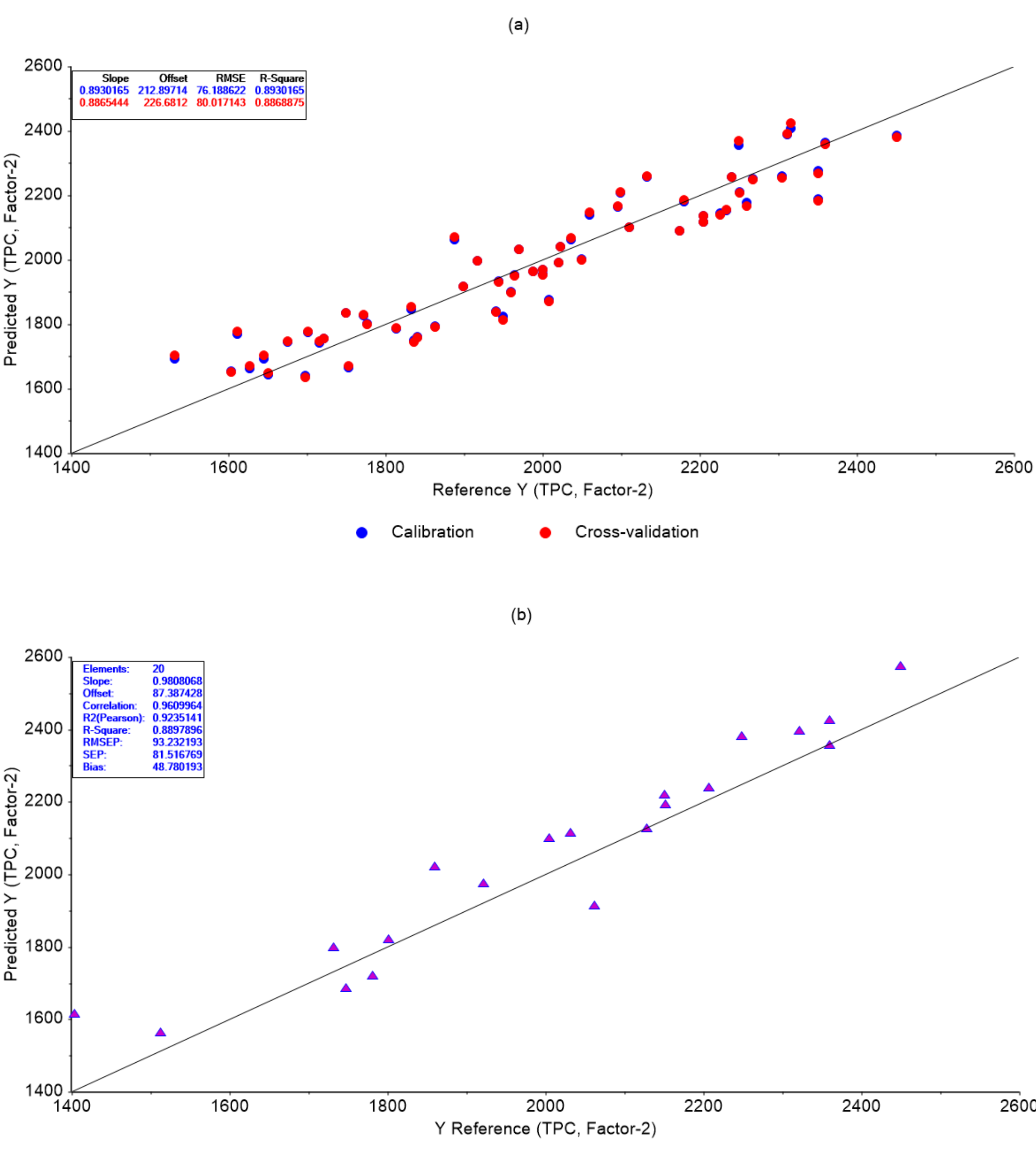

Figure 8-3 (a) Prediction and reference values of TPC (µg GAE/g) for calibration model (60 samples); (b) Prediction values based on calibration and reference values of TPC (µg GAE/g) of external validation data set (20 samples)

TPC: total phenolic content GAE: gallic acid equivalence

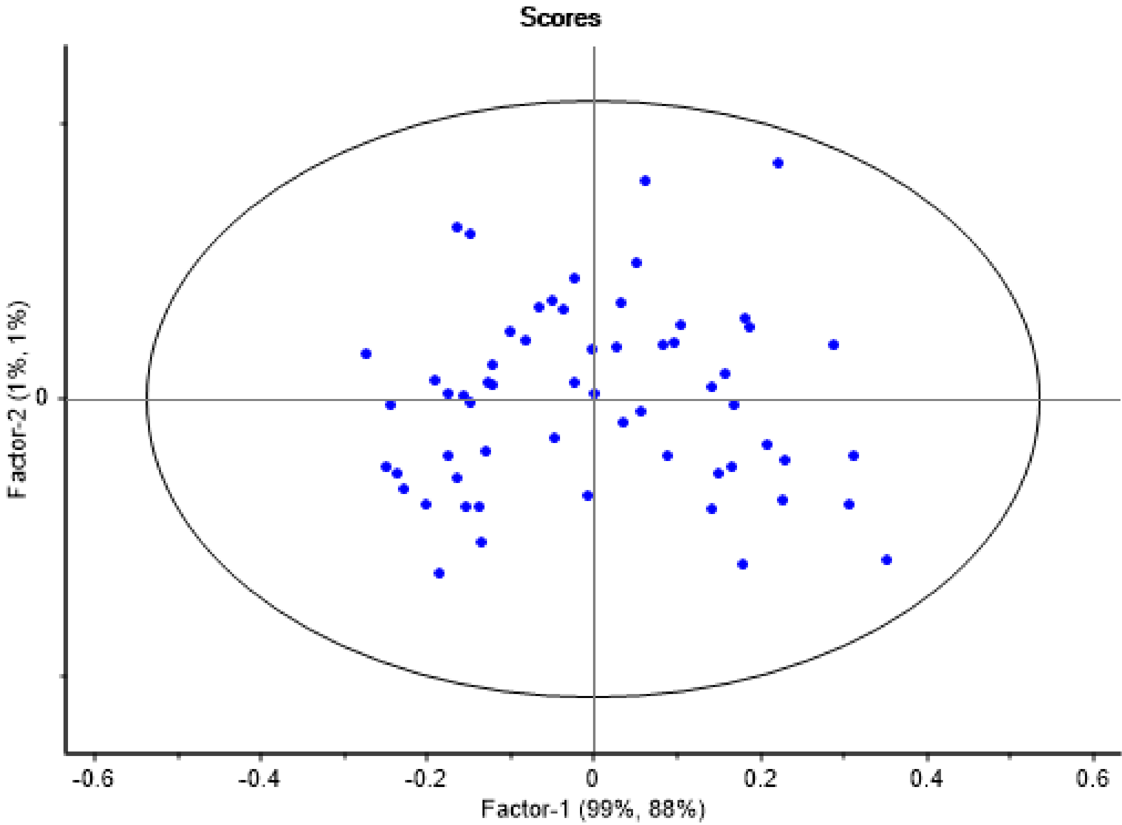

Figure 8-4 Score plot of two major factors from TPC calibration model.

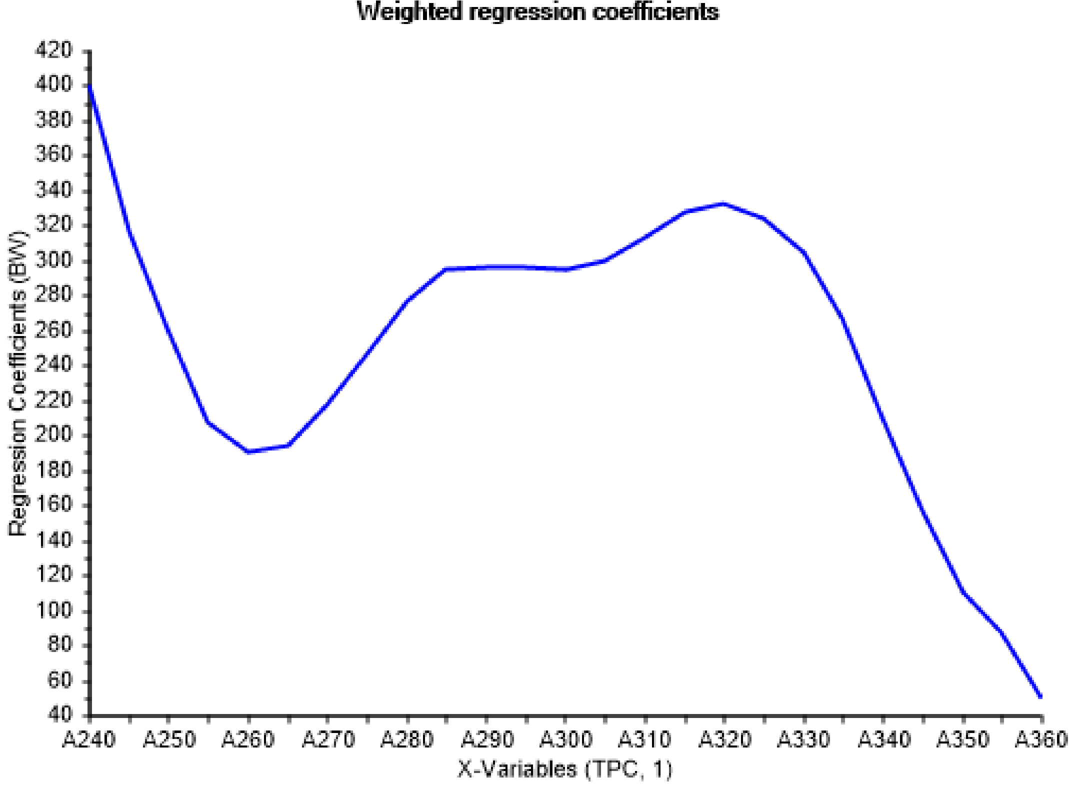

Figure 8-5 Regression coefficient of Factor 1 of TPC calibration model.

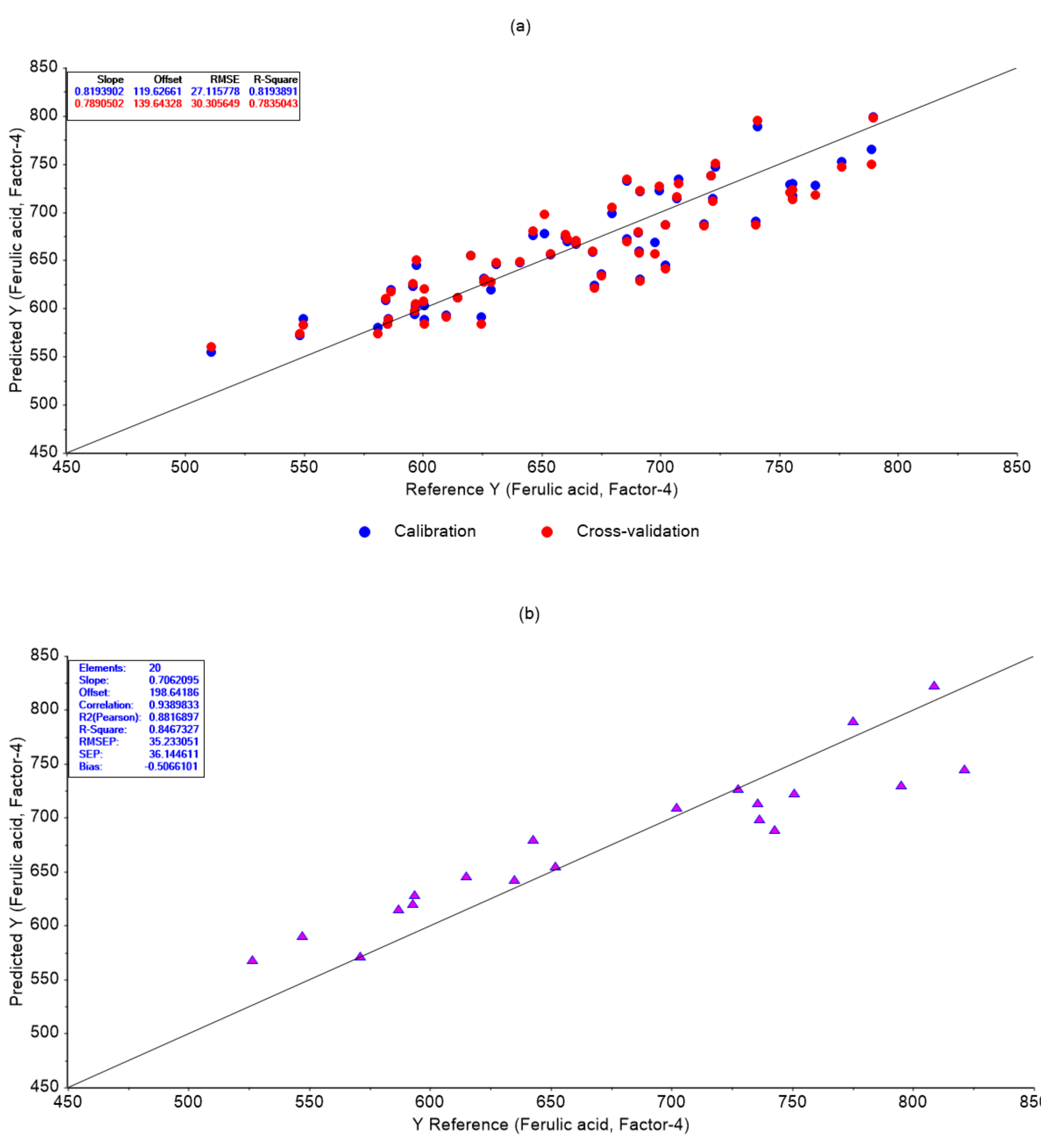

Figure 8-6 (a) Prediction and reference values of ferulic acid (µg/g) for calibration model (60 samples); (b) Prediction values based on calibration and reference values of ferulic acid (µg/g) using external validation data set (20 samples).

Chapter 9 - Rapid determination of total phenolic content of whole wheat flour using near-infrared spectroscopy and chemometrics*

Abstract

Phenolics in whole wheat products provide many health benefits. Wheat breeders, producers, and end-users are becoming increasingly interested in wheats with higher total phenolic content (TPC). Whole wheat flour with higher phenolics may have greater marketing value in the future. However, conventional methods determining TPC are costly and labor-intensive, which are not practical for wheat breeders to analyze several thousands of lines within a limited timeframe. We presented a novel application of near-infrared spectroscopy for TPC prediction in whole wheat flour. The optimal regression model demonstrated R^2 values of 0.92 and 0.90 for the calibration and validation sets, and a residual prediction deviation value of 3.4. The NIR method avoids the tedious extraction and TPC assay procedures, making it more convenient and cost-effective. Our result also demonstrated that NIR can accurately quantify phenolics even at low concentration (less than 0.2%) in the food matrix such as whole wheat flour.

Key words: Near-infrared model; wheat phenolics; Savitzky– Golay derivative; multiplicative signal correction; partial least square regression; rapid screening.

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9.1. Introduction

Growing evidence has indicated that whole wheat products may have the benefits of reducing risks of some chronic diseases and cancers (Jonnalagadda et al., 2011). Wheat phenolic compounds contribute to the health benefits of whole wheat products (Liu, 2007).. Phenolic compounds exist in soluble-free, soluble-conjugated and insoluble-bound forms in wheat kernels, mostly in the bran layers (Adom et al., 2003). These phenolic compounds exhibit potential antioxidant activity, anti-inflammatory effect, and antiproliferative effects (Jonnalagadda et al., 2011). Consumers' desires for food products rich in bioactive compounds have stimulated research on wheat phenolics and their potential health benefits. Wheat breeders and producers are also becoming more interested in wheat lines with enriched phenolic compounds besides conventional targets such as disease and pest resistance, grain yield, protein content, and milling and baking quality. Many different assays have been developed to evaluate the potential antioxidant benefits of wheats. Common assays include total phenolic content (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging, and oxygen radical absorbance capacity (ORAC) assays (López-Alarcón & Denicola, 2013; Tian et al., 2019). HT-29 and Caco-2 human colon cancer cell lines have been used for the evaluation of *in vitro* antiproliferative effect (Lv et al., 2012). TPC assay using Folin- Ciocalteu reagent was most widely used. Though some disagreements exist, TPC is generally positively correlated with other antioxidant assays (Gan et al., 2016) and can serve as the screening method for breeding programs or further analysis such as cellular antioxidant and antiproliferative experiments.

Conventionally, to determine TPC of wheat flour, the major phenolic compounds need to be released by alkaline hydrolysis followed by organic phase extraction and evaporation of the organic solvents (Loreto et al., 2018; Moore et al., 2005; Okarter et al., 2010; Tian & Li, 2018). The sample extract is then analyzed using the Folin- Ciocalteu reagent (Singleton & Rossi, 1965). The whole process is extensively labor-intensive and time-consuming and therefore not effective for screening and selection purposes when hundreds or thousands of samples need to be analyzed. To facilitate further research and applications of wheat bioactive compounds, it is necessary to develop rapid and accurate method for determination of wheat TPC. We previously reported the rapid determination of wheat TPC and ferulic acid by regression models based on UV-Vis spectra of the sample extract (Tian et al., 2020), but that method still requires sample extraction procedures. A more efficient method would be preferred if becoming available.

Near-infrared (NIR) spectroscopy is a non-destructive method and easy to use. Regression models based on NIR spectroscopy have been developed for rapid determination of phenolic compounds in wine (Silva et al., 2014), green tea (Schulz et al., 1999), grape skin (Ferrer-Gallego et al., 2011), sunflower oil (Picouet et al., 2018), mung bean (Meenu et al., 2016), and spent coffee grounds (Magalhães et al., 2016). A successful model usually demonstrated a R^2 value higher than 0.9 and a residual prediction deviation (RPD) value higher than 3.0 (Nicolaï et al., 2007). NIR methods are more effective than traditional methods for screening purposes when large number of samples need to be analyzed. Therefore, the NIR technique is especially useful for wheat breeding programs that usually produce and analyze several thousands of wheat lines each year. However, no NIR application has yet been reported on the prediction of wheat phenolics. This may be because that wheat phenolics are of much lower concentration compared

to tea plants, herbs, and fruits. We hypothesize that NIR spectroscopy can detect structural information that directly or indirectly reflect content of phenolic compounds in whole wheat flour. Proper chemometrics analysis based on NIR spectra can lead to the regression model that is able to predict total phenolic compounds of the whole flour. In this study, we report a PLS regression model based on NIR spectroscopy for rapid determination of total phenolic content of whole wheat flour. Screening and selection process on wheat phenolics from breeding and survey programs, such as the HEALTHGRAIN program (Ward et al., 2008), will be significantly improved by the realization of this technique. To our knowledge, this is the first study developing and reporting an NIR method that utilizes directly whole wheat flours to effectively predict total phenolic content in wheat. Similar regression models based on NIR spectroscopy are also likely to advance the research on phenolic compounds and other phytochemicals in other cereal grains.

9.2. Materials and methods

9.2.1. Wheat samples and chemicals

Hard red winter (HRW) wheats cultivars were grown in Colby, KS and Hays, KS in 2018 and 2019. Randomized complete block design with three replicates was used in the trials. A total of 107 grain samples were collected for this study. Each sample was a blend from its replicated plots. Among this collection, 81 samples were randomly selected for the calibration set, and the remaining 26 samples were used as the validation set. The approximately 3:1 ratio between calibration and validation set has also been employed in previous studies (Wu et al., 2012; Zhang et al., 2017). Wheat kernels were milled to fine flour using a coffee grinder and stored at -20 °C before use. Folin- Ciocalteu reagent for determination of TPC was purchased from Sigma-

Aldrich (St. Louis, MO, USA). Solvents and other general chemicals were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

9.2.2. Extraction of total wheat phenolics

Total wheat phenolics were extracted according to previous studies (Lu et al., 2014) with some modifications. Briefly, one gram of whole wheat flour was extracted with 10 mL ethanol (80%, v/v) for 20 minutes in a 50 mL centrifuge tube, and then mixed with 5 mL NaOH (6M) solution. The mixture was flushed with N_2 gas for 2 minutes and then mixed in a shaker in dark for 3 hours. The slurry then was acidified to pH 2 using concentrated HCl and centrifuged at 4000 g for 30 minutes. The supernatant was collected and extracted with ethyl acetate three times. The combined organic phase was evaporated to dryness, re-constituted to a final volume of 3 mL with HPLC grade methanol in a 5 mL centrifuge tube. The extract was kept at -20 °C and analyzed within 2 days.

9.2.3. Determination of total phenolic content

Total phenolic content was determined according to our previous method (Tian et al., 2019). In brief, phenolic extract of 0.1 mL was mixed with 7.0 ml of deionized water in a centrifuge tube, and then 0.5ml of Folin- Ciocalteu reagent was added. The mixture was vortexed for 5 minutes and then mixed with 1.5 mL Na_2CO_3 (20%, w/v) solution. The final mixture was vortexed and allowed to sit for 2 hours at room temperature for color development. The absorbance at 765 nm was recorded using a VWR UV1600-PC spectrophotometer (Radnor, PA, USA). The final TPC value was expressed as μg gallic acid equivalence (GAE) per gram of whole flour (μg GAE/g) according to a gallic acid standard curve.

9.2.4. Collection of NIR spectra

The NIR spectra were collected using an Antaris II FT-NIR analyzer (Thermo Scientific Inc., Madison, WI, USA) in a reflectance mode. The ground whole wheat flour samples were measured using a sample cup spinner combined with an integrating sphere. Each spectrum was averaged over 64 scans in the wavelength range of 10000– 4000 cm^{-1} at a resolution of 4 cm^{-1}.

9.2.5. Statistical analysis and model development

Original NIR spectra were plotted by TQ Analyst 8.6.12 software (Thermo Scientific Inc., Madison, WI). Spectra pretreatment and the following chemometrics analysis were conducted with Unscrambler 11 software (CAMO Analytics, Oslo, Norway). Principal component analysis (PCA) of collected spectra was used to identify potential outliers. Multiplicative signal correction (MSC) and Savitzky– Golay (SG) derivation were used for spectra pre-treatments. MSC removes the artifacts and imperfections (e.g., scattering effect) from the data matrix before model development. SG derivation alleviates both additive and multiplicative effects in the spectra (Rinnan, 2014; Rinnan et al., 2009).

To develop the regression model, 26 out of the 107 spectra were randomly selected to be the prediction set, and the remaining 81 spectra were used as the calibration set. Full-range spectra (10000– 4000 cm^{-1}) and reduced-range spectra (7500– 4500 cm^{-1}) were used for model development. Spectra ranged from 7500 to 4500 cm-1 were used as the reduced range spectra because most peaks of original and processed spectra (**Figure 9-1**) exist in this range. Partial least squares (PLS) regression was performed for model development. Selection of optimal

number of factors for the PLS model was conducted according to the change of residual variance. The robustness of the model was evaluated in terms of R^2, root mean square error of validation (RMSEV), and residue prediction deviation (RPD).

9.3. Results and discussions

9.3.1. Total phenolic content and NIR spectra

TPC of the tested whole wheat flour samples are provided in **Table 9-1**. The samples of calibration and validation set had comparable mean TPC values and standard deviations. **Figure 9-1** presents the NIR spectra of 107 whole wheat flour samples in the wavelength range of 10000 - 4000 cm^{-1}. The peak at approximately 8300 cm^{-1} was attributed to C-H stretch second overtone (Workman & Weyer, 2012). The peak at approximately 7000 - 6800 cm^{-1} was relatively wide and can be attributed to O- H stretch first overtone of phenolic hydroxyl groups and C-H combination of aromatic structures (Burns & Ciurczak, 2007). The sharp peak at around 5200 cm^{-1} was associated with O-H stretch/ bend of polysaccharides overlapping with water peaks (Burns & Ciurczak, 2007). The peak at around 4800 cm^{-1} might come from asymmetrical C-O-O stretch third overtone of polysaccharides (Zhang, et al., 2017). Though phenolic compounds are generally not considered as the major component in whole wheat flour, characteristics peaks of aromatic compounds were detected, which suggested the potential use of NIR spectroscopy for prediction of total phenolic content of wheat flours.

PCA has been widely used to detect potential outlier spectra and classify NIR data (Xiao et al., 2014). **Figure 9-2** presents the result of PCA principal components. Samples from the calibration set were colored in blue, and samples from the validation set were colored in red. The first

principal component (PC-1) explained 96% of the spectra variation, and the PC-2 explained 3% of the spectra variation. Almost all samples were located within the Hottelling's T^2 ellipse (5%) which suggested that there was no significant outlier spectrum among the 107 tested samples. It indicated that our collected spectra were of good homogeneity. Meanwhile, the variation spectra for both calibration sample set and validation sample set were overall evenly distributed. No apparent difference between calibration spectra and validation spectra was detected. This suggested that our selection of validation sample set was suitable and representative for the model development.

9.3.2. NIR model development

Due to the light scattering and different effective path lengths of solid samples, NIR spectral data inevitably contained some unwanted variations or noise. In order to improve the model development and model accuracy, it is important to perform data pre-processing in the correct manner to reduce these unwanted variations (Rinnan et al., 2009). Scattering correction and spectral derivatives are the most widely used techniques for pre-processing of NIR spectral data. MSC and standard normal variate (SNV) are common scattering methods. MSC and SNV are actually interconvertible and the same for most cases (Dhanoa et al., 1994). In this study, we chose the MSC method. Spectra derivation was performed by Savitzky- Golay (SG) polynomial algorithm. Regression models using full range spectra and reduced range spectra were both developed and compared. Though full range spectra contained more information for tested samples, many studies found that reduced spectra demonstrated better model developments (Xu & Wang, 2013; Zhang, Xu, et al., 2017). This might be because that full range spectra also contained more unwanted variations that can interfere with the regression algorithm. PLS

regression and PCR are common regression algorithms for NIR developments. Our preliminary data (**Table 9-2**) suggested that for this study PLS regression constantly provided better performance than PCR. PLS regression is an algorithm that combines features from principal component analysis and multiple regression. PLS simultaneously decomposes dependent variables (Y) and independent variables (X) to search for components set (called latent vectors or factors) that can explain as much as possible of the covariance between X and Y, in our case, NIR spectrum and TPC. The theorem of PLS has been described in detail previously (Höskuldsson, 1988; Wold et al., 2001).

Table 9-3 summarizes model developments for PLS regression models for wheat TPC. Parameters including R^2, root mean squared error of validation (RMSEV), and RPD which equals to the ratio of standard deviation (SD) and RMSEV were used for the evaluation of model robustness. Higher R^2 and RPD values and lower RMSEV value indicate better model performance. For models using full range spectra, pre-treatments such as MSC and SG derivatives of spectral data showed better model performance than the original spectra. The combination of MSC and first order SG derivative rendered the best model performance with the R^2 calibration of 0.92, R^2 validation of 0.90, and RPD value of 3.4. The average spectrum of the original spectra set and the average spectrum of the processed data set are plotted in **Figure 9-3**. It was clear that spectra pre-treatments led to more sharp peaks in the processed spectra. This might explain why the pre-treatments of the spectra improved the model performance. For original and first order SG derivative, the reduced spectra model did demonstrate better overall performance than the relative full range model. However, for other pre-treatments, the full spectra model was better than the corresponding reduced spectra model. It implied that the

reduced range spectra may not necessarily improve the regression performance. Overall, the best PLS regression model was achieved using the full range spectral data with MSC and SG first order derivative pre-treatments. This model has an RPD values of 3.4. It is generally agreed that NIR model with an RPD value higher than 2.5 is suitable for the prediction of targeted attributes (Martelo-Vidal & Vázquez, 2014).

For PLS regression models, it is vital to determine the optimal number of latent variables (i.e. factors). Though increased number of factors often lead to higher R^2 values, the model is likely to have overfitting issues and not suitable for prediction of future unknown samples (Faber & Rajkó, 2007). Residual variance, or so-called predicted residual error sum of squares (PRESS) in some literatures, was employed to determine the optimal number of factors. In this study, change of residual variance threshold was set at 6% which meant that the change of residual variance had to be higher than 6% to justify the use of an extra factor. **Figure 9-4** presents the plots of residual variance and R^2-validation and number of factors of the PLS model based on MSC and first derivative transformation of the spectral data. The change of residual variance was colored in blue, and the change of R^2-validation was colored in orange. It can be easily observed from the plots that before 7 factors, the residual variance decreased apparently and the R^2 increased apparently. After 7 factors, changes of residual variance and R^2 were negligible. Therefore, 7 factors are the optimal number of factors for the PLS regression model in this study. Factor 1 explained 89% of the X variance. Factor 2 and factor 4 explained 45% and 17% of the Y variance (**Table 9-4**). To show an example of the regression factor, regression factor 2 is plotted in **Figure 9-5**. It can be seen that major positive contributors to factor 2 were approximately at 6750, 5950, 5080, 4650, and 4080 cm^{-1}. There were some wavelength shifts between peaks of the

original spectra and the regression spectrum, which was caused by the Savitzky–Golay first-order derivative treatment. Also, there were wavelength ranges that negatively contributed to the Y variable.

Figure 9-6 presents the regression plot of reference values versus predicted values of wheat TPC. The calibration samples were colored in blue, and the validation samples were colored in red. The results clearly showed that for most data points in either the calibration set or the validation set, the predicted values from the regression model fitted very well with the reference values obtained by the conventional Folin- Ciocalteu method. Values of R^2-calibration and R^2-validation were 0.92 and 0.90, respectively. Similarly, Dykes et al. (2014) reported R^2-calibration and R^2-validation values of 0.97 and 0.86 respectively in an NIR calibration model for determination of sorghum total phenols. Wiedemair et al. (2019) reported an NIR model for determination of TPC of gluten-free cereals with a R^2-validation of 0.88. To the best of our knowledge, our study is the first NIR model for a rapid determination of wheat total phenolic content.

9.3.3. Cost-benefit analysis compared to the conventional method

Most of the wheat phenolics exist in insoluble-bound fractions. Conventionally, wheat flour is hydrolyzed by alkaline treatment, acidified, centrifuged, and extracted using organic solvent (such as ethyl acetate), and majority of the solvents need to be further evaporated to concentrate the phenolic solution. Though the detailed protocols may vary at different laboratories, typically 1 g of whole flour is hydrolyzed by 10 mL 2M NaOH solution and acidified by concentrated HCl. The organic extraction process normally consumes 50 mL ethyl acetate for each sample. It

also takes a substantial amount of time to evaporate the organic solvent. After sample extraction, the TPC is determined using Folin- Ciocalteu regent. Though TPC assay has been widely used, it requires good lab skills in order to obtain reproducible results. Based on our estimation, an experienced lab technician can at most analyze 25 samples with replicates in a week. Survey or breeding programs with hundreds or thousands of samples would take several months to complete the analysis. However, a technician could conduct hundreds of TPC analysis with the new NIR method with comparable accuracy in a much shorter time. The cost of chemicals, hazardous waste and especially labor would be greatly reduced.

9.4. Conclusions

In summary, we developed an effective PLS regression model for rapid determination of total phenolic content in whole wheat flour based on NIR spectroscopy and chemometric analysis. Compared with conventional wet chemistry methods, the NIR model is more efficient, eco-friendly and less labor-intensive. Wheat breeding programs and other programs that require fast screening and selection of wheat lines with enriched phenolic compounds will greatly benefit from this new technique.

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recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Conflict of interest

The authors declare that there is no known conflict of interest.

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Figures and tables

Table 9-1 Statistics of total phenolic content of the tested whole wheat flours using reference method (i.e., Folin-Ciocalteu assay).

	Range (µg/ GAE g)	Mean (µg/ GAE g)	Standard Deviation (µg/ GAE g)
Full Set	1403.0- 2451.0	1975.7	238.8
Calibration Set	1403.0- 2451.0	1977.4	243.5
Validation Set	1611.0- 2360.0	1970.5	227.8

GAE: gallic acid equivalence

Table 9-2 Comparison between partial least squares (PLS) regression and principal component regression (PCR).

	Original spectra	MSC	First derivative	MSC+ first derivative	Second derivative	MSC+ Second derivative
PLS with full range spectra						
R^2, calibration	0.78	0.87	0.89	**0.92**	0.93	0.95
R^2, validation	0.71	0.77	0.85	**0.90**	0.82	0.82
RMSEV	119.6	106.1	85.6	**70.5**	96.1	95.7
RPD	2.0	2.3	2.8	**3.4**	2.5	2.5
Factors used	14	14	8	**7**	4	4
PCR with full range spectra						
R^2, calibration	0.55	0.52	0.73	0.73	0.68	0.69
R^2, validation	0.48	0.49	0.63	0.64	0.60	0.59
RMSEV	176.4	176.0	150.5	148.4	154.1	156.2
RPD	1.5	1.5	1.8	1.8	1.7	1.7
Factors used	6	3	11	10	7	8

MSC: Multiplicative signal correction; RMSEV: Root mean square error of validation; RPD: Residual prediction deviation.

Table 9-3 PLS regression models for wheat total phenolic content prediction.

	Original spectra	MSC	First derivative	MSC+ first derivative	Second derivative	MSC+ Second derivative
Full Spectra (10000- 4000 wavenumbers cm $^{-1}$)						
R^2, calibration	0.78	0.87	0.89	**0.92**	0.93	0.95
R^2, validation	0.71	0.77	0.85	**0.90**	0.82	0.82
RMSEV	119.6	106.1	85.6	**70.5**	96.1	95.7
RPD	2.0	2.3	2.8	**3.4**	2.5	2.5
Factors used	14	14	8	**7**	4	4
Reduced Spectra (7500- 4500 wavenumbers cm $^{-1}$)						
R^2, calibration	0.72	0.76	0.90	0.92	0.90	0.91
R^2, validation	0.76	0.76	0.88	0.88	0.79	0.80
RMSEV	110.7	109.7	78.5	78.5	103.4	99.1
RPD	2.2	2.2	3.0	3.0	2.3	2.4
Factors used	12	12	8	7	4	4

MSC: Multiplicative signal correction; RMSEV: Root mean square error of validation; RPD: Residual prediction deviation.

Table 9-4 Contributions of regression factors to explained variance of Y variable and X variables.

	Percentage of contribution to X variables	Percentage of contribution to Y variables
Factor 1	89%	5%
Factor 2	3%	45%
Factor 3	5%	6%
Factor 4	0%	17%
Factor 5	1%	7%
Factor 6	1%	3%
Factor 7	0%	9%
Total	100%	92%

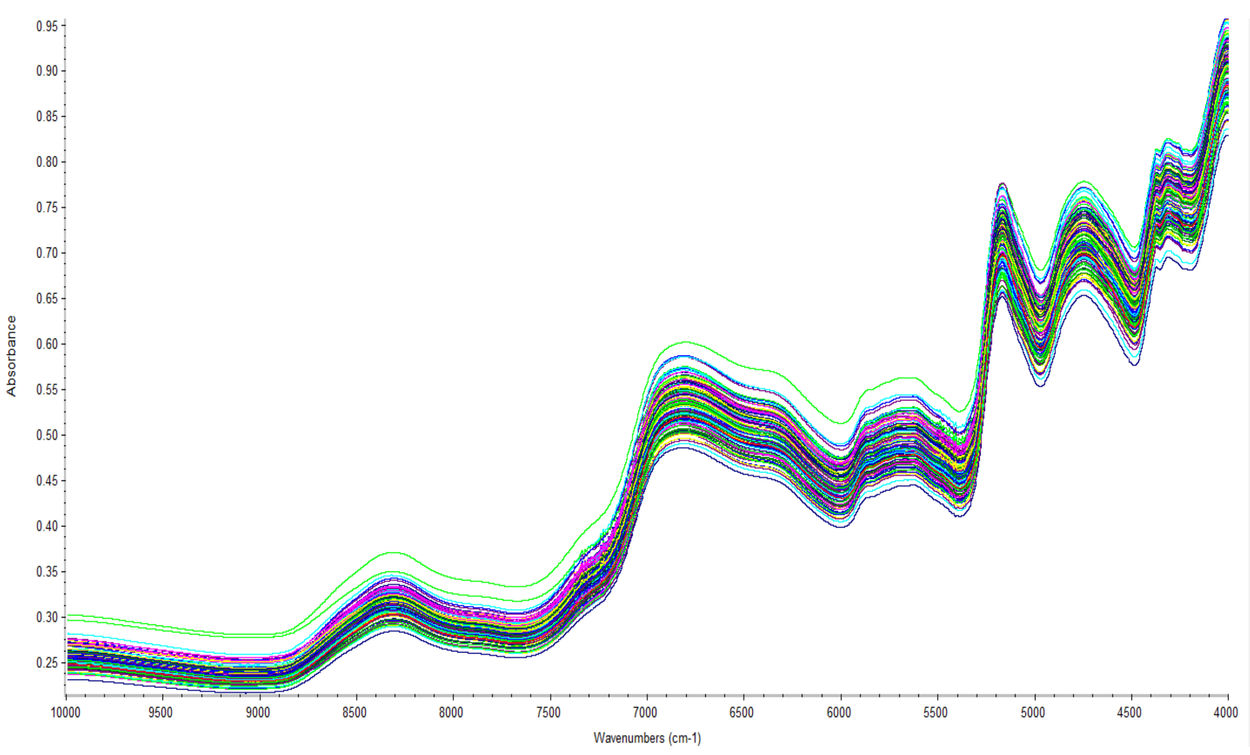

Figure 9-1 NIR spectra of whole wheat flours at a resolution of 4 cm−1 ranged from 10000 to 4000 cm^{-1}.

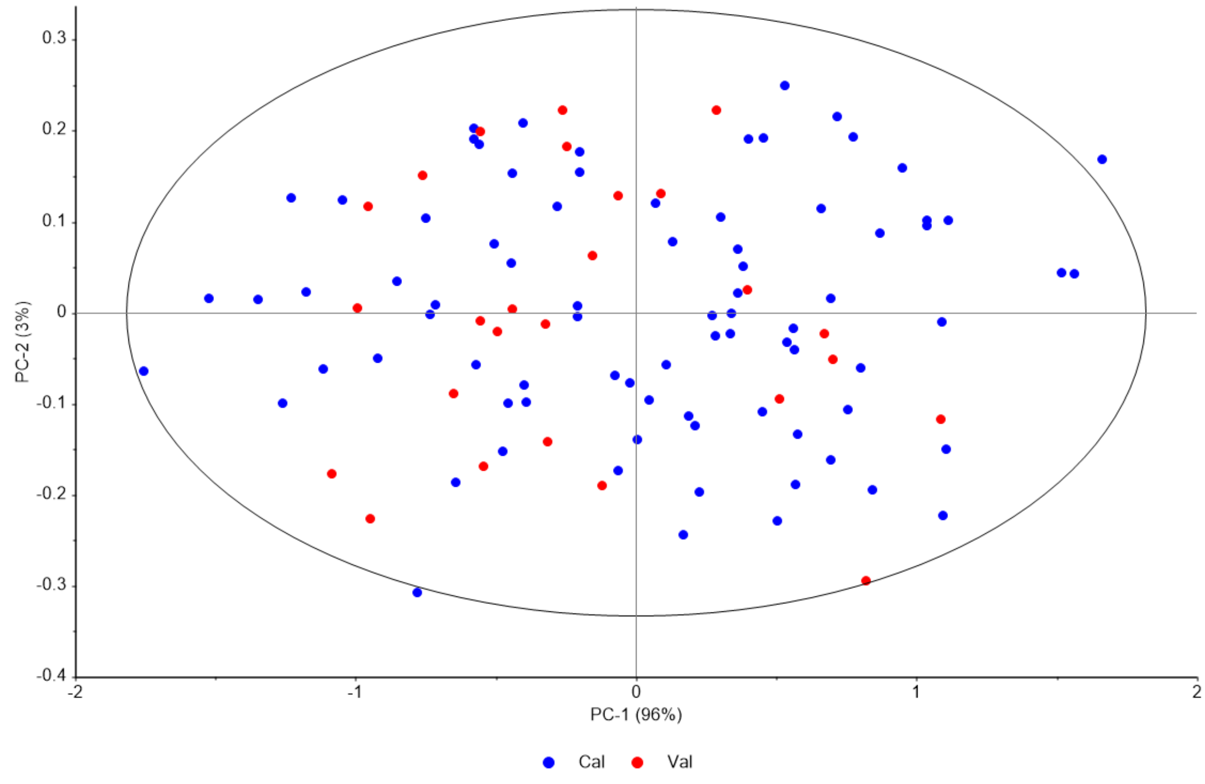

Figure 9-2 Principal component analysis (PCA) of whole wheat flour spectra.
Blue dots represent samples in calibration set (81 samples); red dots represent samples in validation set (26 samples). Hotelling's T^2 ellipse (5%) are used to identify outlier spectra.

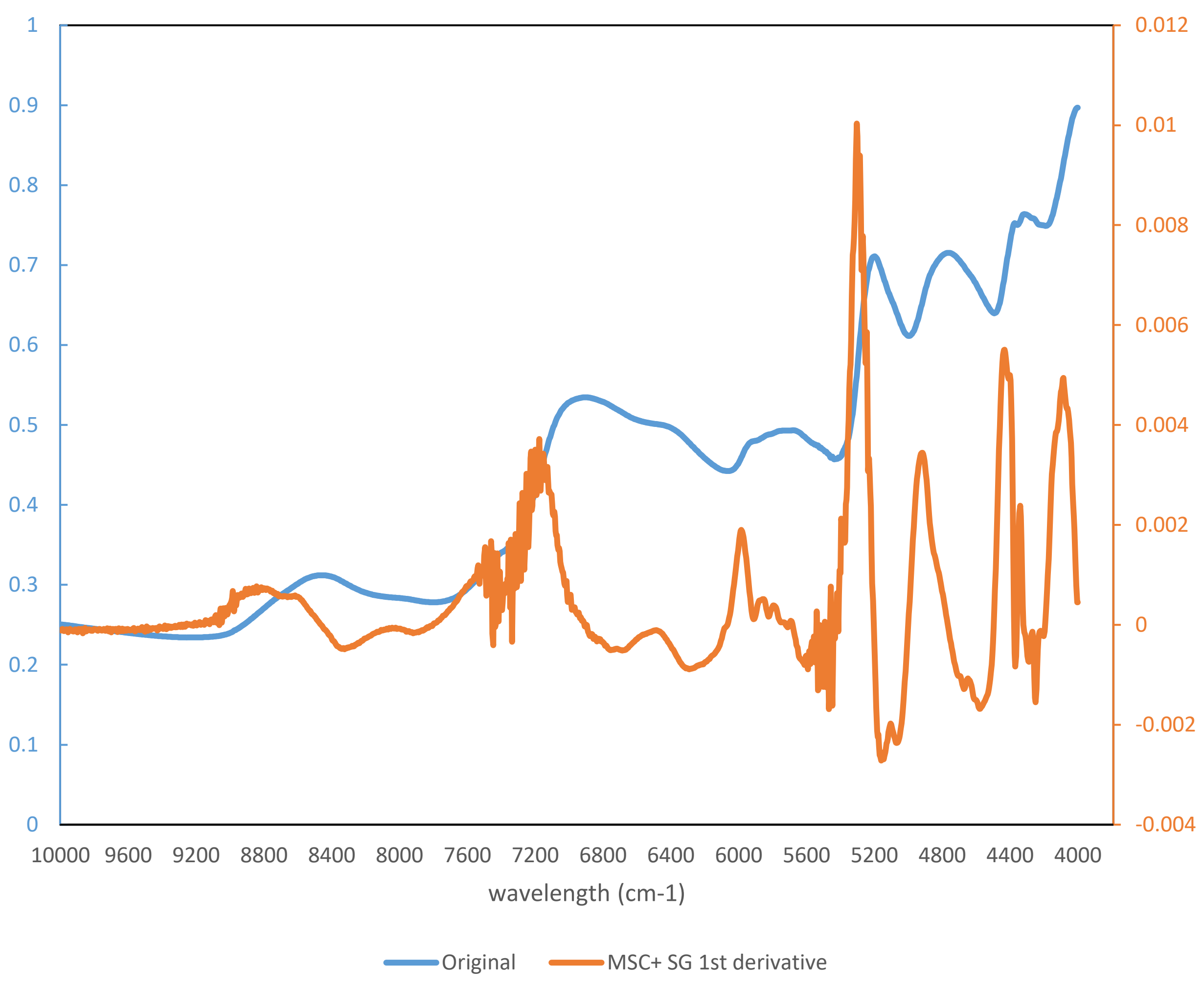

Figure 9-3 . Comparison of averaged spectrum of the original spectra set (blue) and averaged spectrum of pre-processed spectra set (orange).

MSC: Multiplicative signal correction; SG: Savitzky–Golay.

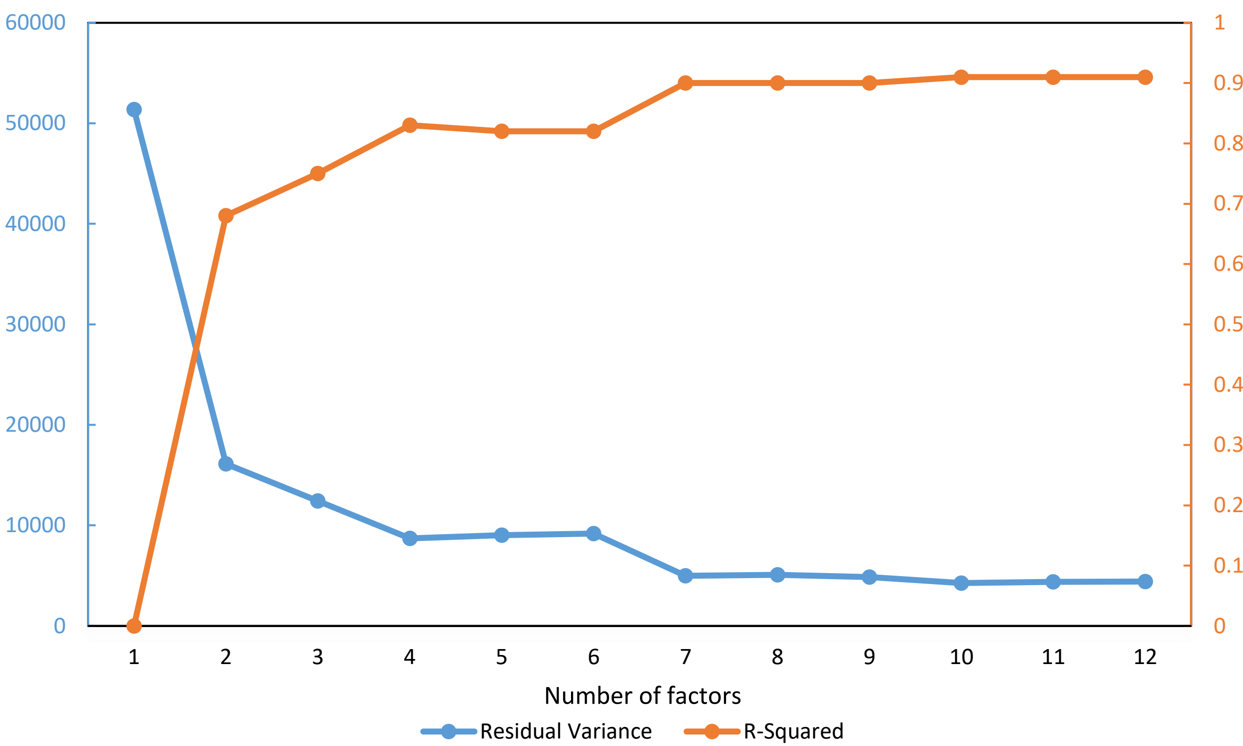

Figure 9-4 Residual variance and R-squared plots of calculated number of factors in PLS prediction model based on MSC and first derivative transformation of the spectral data.

The blue axis on the left represents residual variance; the orange axis on the right represents R-squared value.

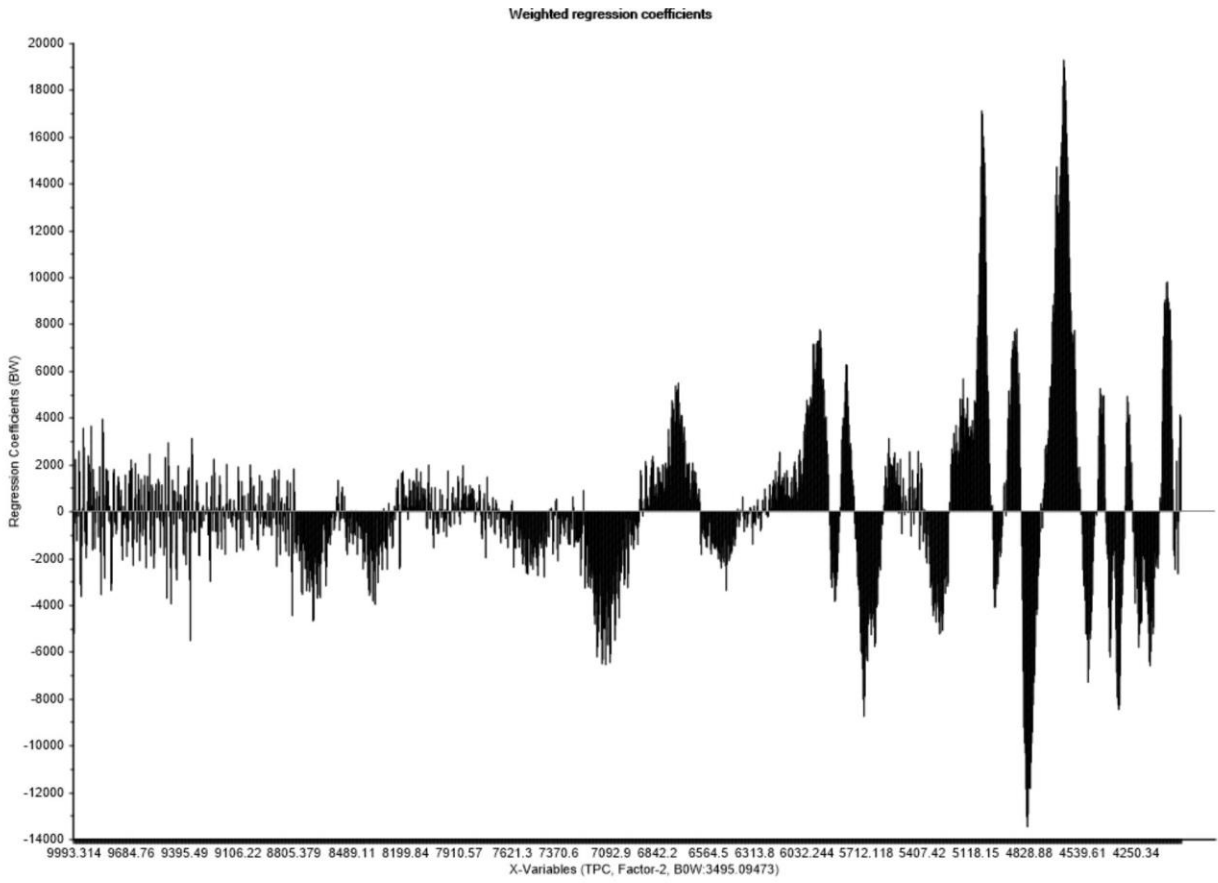

Figure 9-5 Plot of the regression factor 2 in the developed model.

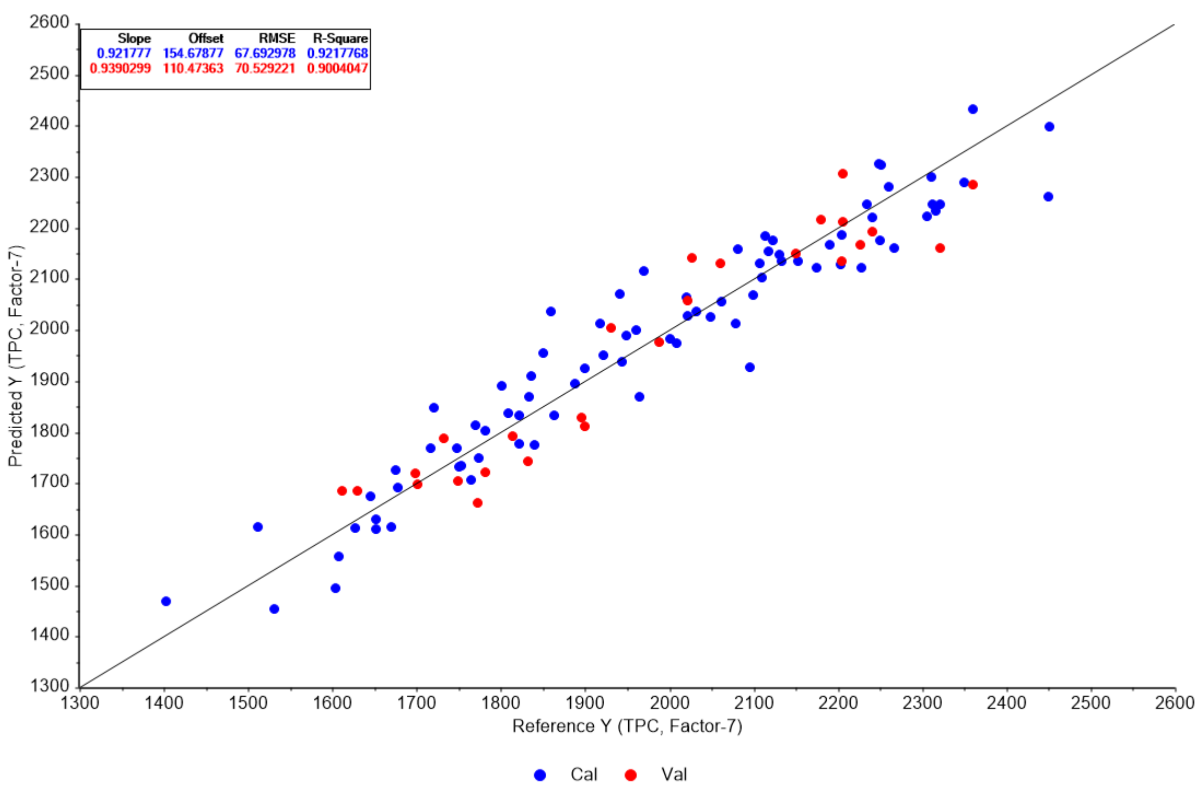

Figure 9-6 PLS regression plot of reference values versus predicted values of wheat total phenolic content (TPC).

Blue dots represent samples in calibration set; red dots represent samples in validation set.

Chapter 10 - Conclusions and future perspectives

Wheat is among the most important cereals for humanity. The health benefits of whole wheat foods can partially be attributed to the phytochemicals that mainly exist in wheat brans. In recent years, driven by humanity' desire for healthier food sources, potential health benefits of wheat grains have become significant quality parameters. Phenolic acids are a major group of phytochemicals in wheat and exist in soluble-free, soluble-conjugated, and insoluble-bound forms. This study aimed to understand the changes of phenolic acid profiles and antioxidant potential of wheat products in the wheat food chain (e.g., breeding and genotype development, field management and fertilization, early-stage grain germination, bread-making, and *in vitro* gastrointestinal digestion and probiotic fermentation) and develop novel spectroscopy methods for rapid quantification of total phenolics in whole wheat.

Previous studies related to the topic were extensively reviewed in Chapter 1. Chapter 2 described phenolic profile and antioxidant activities of 12 common Kansas hard red winter wheat varieties. The results indicated that concentrations of phenolic acids and antioxidant potential varied greatly among wheat genotypes, caused by genetical differences. Chapter 3 described interaction effects of environment, wheat genotype, and fertilizer levels on phenolic profile and antioxidant activities of whole wheat flours. Chapter 4 described effects of integrated wheat management strategy on phenolic concentration of wheat brans. The results showed that farming practices for improved yield and wheat quality also significantly affected the accumulation of wheat phenolics. Year x management interaction was significant; therefore, farmers are advised to

adjust farming strategies based on market preference and weather conditions. Chapter 5 described effects of early-stage seed germination on both baking properties and phenolic profiles of multiple winter wheat varieties. The results suggested that early-stage germination (<24 hours) decreased concentration of phenolic acids. Germinated flour generally retained comparable bread-making properties. Chapter 6 described changes of phenolic acid profile and antioxidant activities during bread-making process including mixing, fermentation, and baking. Fermentation and baking had some positive effect on the phenolic profile. UPLC-DAD-ESI-Q-TOF-MS/MS analysis showed that some phenolic acids can be incorporated into Maillard reactions products. Chapter 7 presented changes of phenolic acids and their potential bio-accessibility during simulated gastrointestinal digestion and probiotic fermentation. Digestive enzymes can release phenolic acids from insoluble-bound forms while major part of *trans*-ferulic acid can reach the colon. Chapter 8 and chapter 9 presented two newly developed rapid quantification methods for wheat phenolics. Chapter 8 showed models for rapid quantification of total phenolic content (TPC) and concentration of *trans*-ferulic acid based on UV absorbance spectra. To further simply the process, in Chapter 9, we presented a NIR model that can accurately and rapidly determine TPC of whole wheat flour. Modeling methods greatly reduced cost of screenings for wheat genotypes with enriched phytochemical content. In summary, this study covers many aspects of wheat chains regarding wheat antioxidants and provides new insights towards the understanding of wheat phytochemicals that will benefit both academic research and wheat industries.

Besides topics that have been described in this thesis and many previous studies, the following fields have not been extensively studied but may worth further investigating in the future: 1)

development of new, standardized extraction protocol for enhanced extractability of wheat phytochemicals; 2) in-depth structure characterizations by advanced analytical instrument such as solid-state NMR on the network between phytochemicals and cell wall materials; 3) relations of wheat phytochemicals and conventional parameters evaluating wheat qualities such as grain yield, protein content and end-use properties; 4) bio-markers of whole grain intake; 5) bioaccessibility, bioavailability, metabolisms and prebiotic effect of wheat phytochemicals in the human body.