The Pennsylvania State University

The Graduate School

College of Agricultural Sciences

ELUCIDATING DIFFERENCES IN PHENOLIC PROFILE BETWEEN TEF (*ERAGROSTIS TEF)* VARIETIES USING MULTIVARIATE ANALYSES

A Thesis in

Food Science

by

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

August 2017

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ABSTRACT

Tef (*Eragrostis tef)* is a cereal grain endemic to Ethiopia. Due to its small size it is milled whole and, therefore, tef flour retains the phenolic compounds that are present mostly in the bran layer of the grain. These phenolic compounds are found in three forms: free, conjugated (bound to soluble fiber/sugars), and bound (bound to insoluble fiber). In cereals, bound phenolic compounds make up the largest portion of total phenolics and can be released by hydrolyzing the ester bonds with strong alkali or acid. It is thought that the distribution of phenolic compounds between these fractions and interaction with fiber within a certain food can have unique influence on metabolism and physiology in the body of those who consume it. However, many factors including variety can affect phenolic content. Eight tef varieties, six grown in the same season (2012) and location (Bishoftu, a.k.a. Debrezeit, Ethiopia) and two grown in Idaho, USA (purchased from TeffCo in 2016) were extracted to characterize their free, conjugated, and bound phenolic profiles. Extracts were analyzed by high performance liquid chromatography with a diode array detector (HPLC-DAD), and phenolic acids were identified and quantified by matching peak elution times to external standards and standard calibration curves, respectively. Principal component analysis (PCA) and multifactor analysis (MFA) were run to compare chromatograms of the different fractions and varieties. These analyses were able to differentiate between brown and white varieties in the free and bound phenolic fractions with separation in the first principal component being dominated by large hydrophobic compounds eluting in the flavonoid portion of the chromatogram. Ferulic acid was found to be the most abundant phenolic compound in the bound fraction with the DZ-Cr-384 (Kuncho) variety having the highest ferulic acid content (280.73 μg/g defatted tef flour). There were no significant differences in total phenolic content between varieties as determined by the Folin-Ciocalteu assay.

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ACKNOWLEDGEMENTS

I would first and foremost like to thank my advisor and mentor Dr. Gregory R. Ziegler for the opportunity to come to Penn State and conduct this research. I greatly appreciate your guidance and support over these past two years. I am grateful for all that I have learned through this experience and I have enjoyed working in your lab.

Thank you also to Dr. Joshua D. Lambert and Dr. Jairam K.P. Vanamala for serving as members of my committee and for all of their advice and support in my research. Special thanks to Dr. Ramaswamy C. Anantheswaran and Dr. Ryan J. Elias, for allowing me to use their labs and equipment for experiments. Thank you to Dr. Helene Hopfer for all of your patience and advice regarding statistics and analytical methods. Special thanks to Dr. Iffa Gaffoor and Dr. Surinder Chopra for all of your guidance and for allowing me to grow my tef the greenhouse. Thank you to the Penn State University Food Science Department Faculty and Staff for a great graduate school experience. I enjoyed interacting and working with you these past two years. Special thanks to Svend Pedersen for all your help with scheduling, enrollment, and administrative deadlines. Also, thank you to my mentor Dr. Aynur Gunenc, for all of your advice and for helping me settle into research and graduate school.

I would like to thank my lab mates Hui Wang and Jason Wang, my roommates Qiaoqiao Dai and Michelle Tran, and all of my friends in the department for your encouragement and friendship these two years. To my brother Adam, to Katya, Nikita, Luciana, and Sam, thank you for the long-distance phone calls and visits. Special thanks to Ben Kass for your constant encouragement, support, and humor. Finally, thank you to my parents. You have always encouraged my love for learning and science, and you have been my role models in all that I do.

1. Introduction

The U.S. Department of Agriculture, in its release of the *2015-2020 Dietary Guidelines for Americans*, recommends that whole grains make up half of the total grains in the daily consumer diet (USDA 2015). Consumers are becoming more interested in whole grain products because they are associated with lowered risk of chronic diseases such as heart disease, colon cancer, and type II diabetes (Dykes and Rooney 2007). This is mainly due to the health benefits attributed to whole grains which retain the vitamins, minerals, and dietary fiber lost upon refining (Whole Grains Council 2013). Concurrently, research on dietary phenolics has centered on exploring potential anti-inflammatory and anti-cancer health benefits of these plant-derived compounds and their metabolites (Russell et al. 2006; Monagas et al. 2009). Phenolic compounds exist in many forms, including free, glycosylated or bound to soluble fiber (conjugated), and bound to insoluble fiber (bound). It has been shown that the majority of these compounds are found in the bran portions of the grain and are esterified to components of dietary fiber (Kim et al. 2006; Chandrasekara and Shahidi 2010).

In their review of the role of dietary fiber in human health, Vitaglione, Napolitano, and Fogliano (2008) build upon several studies to develop a hypothesis that it is the continued release of conjugated and bound phenolic acids in the gut by bacterial enzymes, and their subsequent absorption into the blood stream, that confers the health impact long associated with whole grain and bran consumption. Consequently, research that determines the phenolic profile of grains, i.e. how phenolic compounds are distributed between the three forms within foods, can provide a good foundation for future whole-food centered research.

Major grains such as wheat, barley, maize, and oats have been studied for their phenolic profiles, as well as the effects of processing and factors such as variety and growth conditions on

their phenolic composition (Moore et al. 2007; Verma, Hucl, and Chibbar 2008; Zhang et al. 2010; Hole et al. 2012; Guo and Beta 2013;Shao et al. 2014). Phenolic composition of grains such as sorghum, millet, and the pseudo cereal quinoa which are staples in diets across the world (India, Africa, South America, respectively) have also been studied (Chandrasekara, Naczk, and Shahidi 2012; Tang et al. 2015; Taylor and Duodu 2015), and with increased interest in whole-grain intake and diversification of crops, as well as the popularization of 'ancient grains,' they are entering the wider world market (Cherfas 2015; Romer 2015; Shipman 2016).

The grain derived from tef [*Eragrostis tef* (Zucc.) Trotter] is poised to follow a similar trajectory. Tef, originating in Ethiopia, at an average of 0.264 mg per seed, is reported to be the smallest cultivated grain in the world and, as such, it is milled or consumed as a whole-grain, retaining its bran and germ (Bultosa 2007; Baye 2014). It has a nutritional profile comparable to grains like wheat, has high fiber content (Baye 2014), and is resilient to a variety of growing conditions (Assefa et al. 2010). Considerable research on tef cultivation, macronutrient content, as well as starch properties (as tef is predominately consumed as a fermented pancake-like bread called *injera)* has been conducted (Bultosa, Hall, and Taylor 2002; El-alfy, Ezzat, and Sleem 2012; Assefa, Chanyalew, and Tadele 2013; Baye 2014; Cheng et al. 2015). However, research on tef's phenolic profile is limited, with only a few studies providing quantitative identification of phenolic compounds, too few to provide sufficient detail of tef's phenolic profile (El-alfy, Ezzat, and Sleem 2012; Boka, Woldegiorgis, and Haki 2013; Salawu, Bester, and Duodu 2014; Shumoy and Raes 2016; Sumczynski et al. 2016; Shumoy et al. 2017). Extant studies differ so greatly in methodology and sample sets that more research is needed to fill gaps concerning the influence of variety (while controlling for location) and pigmentation differences on phenolic composition. Greater focus on tef's phenolic profile will inform future studies of its physiological impacts on human health, as studies of this nature are of limited reach, often being based on association of nutritional profile with potential positive health effects (Hopman et al. 2008; Baye 2014).

Therefore, this work aims to analyze the effect of variety on the phenolic profile of tef grain (*Eragrostis tef*) by extracting polyphenols from six tef varieties that were grown in the same location and season in Ethiopia, along with two U.S. grown commercial varieties for comparison. High performance liquid chromatography (HPLC)-based quantification and subsequent principal component analysis (PCA) and multifactor analysis (MFA) were used to elucidate similarities and differences in the phenolic profiles across three extract fractions, i.e. free, conjugated, and bound, and several assays were employed to explore the pigmentation of tef grains, with the goal of identifying the compounds responsible for the color of some varieties.

2. Literature Review

2.1 Tef as a Cereal Crop

Tef (*Eragrostis tef* (Zucc.) Trotter) is a staple cereal crop endemic to Ethiopia and, at 0.264 mg per seed, is often cited as the smallest cultivated grain in the world. It is thought to have been domesticated between 4000-1000 B.C. (Bultosa 2007; Tesema 2013). Tef is grown on 2.8 million hectares by over 5.6 million farmers in Ethiopia to feed 50 million people in the country (Central Statistical Agency 2010). Tef is also grown in countries outside of Ethiopia, such as the United States, but it remains a niche crop. Since 2007 the Ethiopian government has banned the export of tef grain from Ethiopia, with only recent allowances for the export of tef flour abroad (Ferede 2013; Nurse 2015; Sanchez 2015).

2.1.1 Tef Production

In Ethiopia, tef is planted and grown in the rainy season (July – October) (Food and Agriculture Organization of the United Nations 2017). Prior to row planting, or more traditional broadcasting, seedbeds are tilled repeatedly and then made smooth by trampling, usually with animals like cattle or sheep (Kelemu and Kebede 2013). The grain is traditionally harvested with a hand-held sickle, however varieties with improved lodging resistance allow for increased application of mechanical harvesting (Kelemu and Kebede 2013). Upon harvest, stalks are scattered on a beaten down plot of dirt and domestic animals are brought in to trample on the stalks to thresh the loosely attached seeds (Bultosa and Taylor 2004). Mechanical tools for all stages of tef production, including threshing, are being introduced and improved. Fufa et al. (2011) reports that mechanical threshing can reduce grain losses by 0.2 tons/hectare (Kelemu and Kebede 2013).

Threshed grain is then winnowed, usually using wind or fanning, to separate the chaff. Grains may also be sieved to remove dirt and particulates before storage. An advantage of tef is that upon dry storage it is resistant to most storage pests and therefore has a longer shelf-life than other grains (Refera 2001). Finally, tef is milled by hand with the *wefcho*, which is a set of grinding stones, or various types of mechanical/electric mills (Refera 2001).

2.1.2 Tef Varieties and Research

Tef has vast genetic variability in Ethiopia, the center of origin and diversity (Vavilov 1951 as cited in Chanyalew, Assefa, and Metaferia 2013). This diversity lends itself well to breeding the crop to withstand changes in climate, improve crop nutrition, increase yield, and contribute to farmer income by producing grain of favorable quality (Chanyalew, Assefa, and

Metaferia 2013). The crop is already known for its versatility, being cultivated at altitudes between 800-3200 meters above sea level, in both drought-prone and rainy areas (Tesema 2013). Since the 1950's there have been an increasing number of tef breeding projects and programs aimed at increasing tef productivity (yield, lodging resistance, low-moisture tolerance) and seed quality, with a preference for white varieties due to the high marketability and productivity (Assefa 2013). As a result of these efforts there are now 32 improved varieties grown in Ethiopia (Ferede 2013). Currently, the Kuncho (Quncho; DZ-Cr-384) variety is heralded as the greatest success of these programs, as it is a hybrid of the very-white seeded Magna (DZ-01-196) and high yielding Dukem (DZ-01-974) varieties (Assefa et al. 2011; Ferede 2013).

2.1.3 Tef Consumption and Potential Health Benefits

Consumption

Tef is most commonly consumed in the form of a fermented, pancake-like bread called *injera*, but can also be made into porridge, beer or spirits (Umeta and Faulks 1988). This gluten-free grain is gaining recognition outside of Ethiopia, both due to the increased export of tef flour to meet the demands of the Ethiopian diaspora, but also because of its nutritional profile. Tef's amino acid profile compares well to gluten-containing grains such as wheat and barley (Bultosa and Taylor 2004; Gebremariam, Zarnkow, and Becker 2012), and tef contains almost four times the amount of calcium found in grains such as barley, wheat, rye, maize, sorghum, and pearl millet (Gebremariam, Zarnkow, and Becker 2012).

Anemia, Celiac's Disease, Osteoporosis, and Diabetes

Initial nutritional studies on tef determined that it contained high levels of iron, and it was thought that this was why populations consuming tef in Ethiopia had lower incidence of anemia (Costanza, DeWet, and Harlan 1979). However, further study demonstrated that the high iron levels associated with tef are likely due to soil contamination during harvest and threshing, and that iron analysis after thorough cleaning reveals similar iron levels to most cereal grains (5 mg/g) (Besra, Admasu, and Ogbai 1980; Mamo and Parsons 1987).

As a gluten-free grain, tef is a viable cereal alternative for people with Celiac's disease. A Dutch study found a three-fold reduction of the self-reported symptoms of Celiac patients when tef was added to their gluten-free diet (Hopman et al. 2008). The authors note that the study was preliminary and not meant to elucidate cause and effect, but also viewed this observed reduction as a potential dietary impact of tef on Celiac symptoms that merits further study. While the direct effects of tef as a part of a gluten-free diet are not well described, another prospective benefit of tef lies in its nutrient profile. Unlike other gluten-free cereals, tef is more nutritionally similar to cereals like wheat and therefore substituting tef for another gluten-free grain adds value for the consumer. Moreover, as Baye (2014) contends, avoidance of common cereal grains, when most gluten-free alternatives have limited nutritional benefit, results in nutrient deficiencies that can potentially be ameliorated with an introduction of tef into the diet.

Baye (2014) also suggested that tef can potentially help control diabetes, as it has a lower glycemic index (GI) than wheat. Wolter et al. (2013) estimated that tef bread has a lower glycemic index than wheat bread (74 vs. 100 respectively) and would, therefore, be a good alternative to wheat bread. However, Shumoy and Raes (2017) did not recommend tef, in the form of *injera* or tef porridge for consumption as a lower GI food for diabetics. Shumoy and Raes (2017) found no relationship between tef varieties with higher levels of resistant starch and

those that had lower estimated GI values, though resistant starch is often considered a marker for lower GI. The estimated GI values reported for various tef varieties ranged from 79-99 and 94-137 for porridge and *injera,* respectively (with white bread as reference). While both studies reported that tef products were in the mid to high GI range, Shumoy and Raes (2017) attributed differences between the studies to the type of processing method used, since bread likely has a lower water content than porridge. Also, the starch profile of injera is significantly altered by fermentation which can influence digestibility (Umeta and Faulks 1988).

Without further studies on tef intervention in the diet, it is still unclear whether tef is an ideal addition to the diet of people who already have diabetes. However, substitution of tef for higher GI grains could potentially help prevent development of diabetes in at-risk patients. Because it is consumed as a whole grain, it has high levels of dietary fiber and also contributes phenolic compounds that could influence inflammatory responses responsible for the onset of insulin resistance and Type-2 diabetes (Baye 2014).

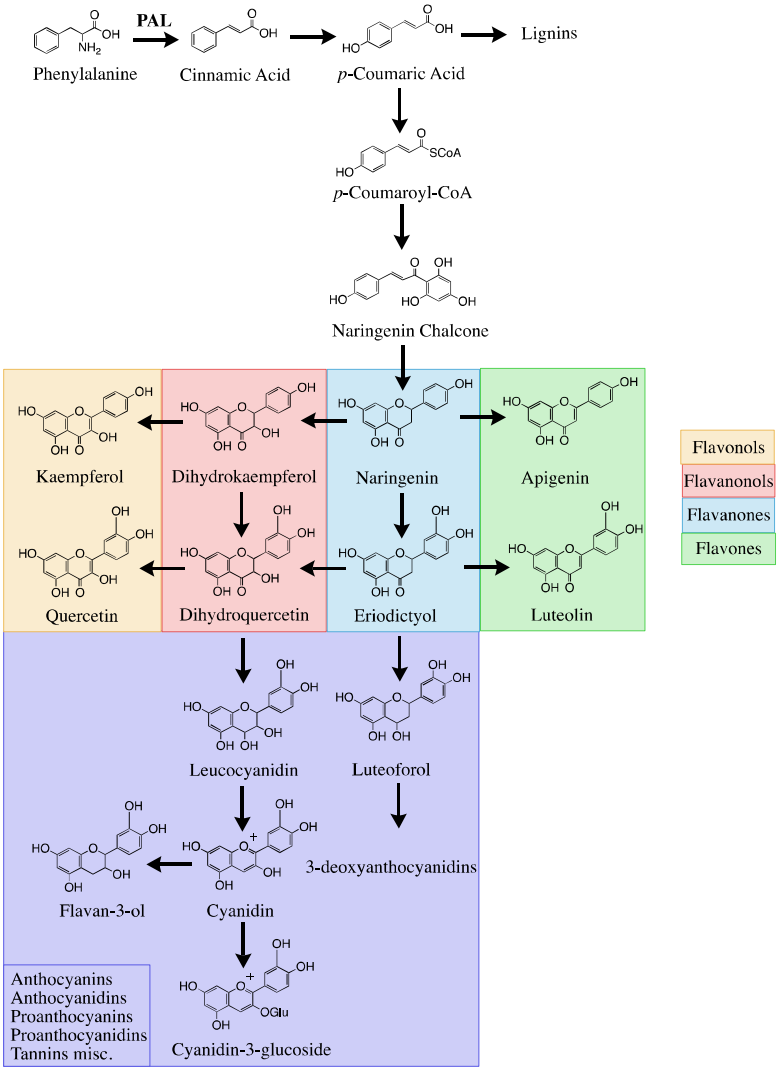
There has been little *in vitro* or *in vivo* research on tef's potential health benefits. However the benefits of whole grains are attributed to the fact that they are consumed without removing any portion of the grain, including the bran and germ, which are a good source of dietary fiber and phenolic compounds (Tuohy et al. 2012; Whole Grains Council 2013). In order to elucidate the influence of whole grain-derived phenolic compounds on health, it is first important to understand what phenolic compounds are present and in what form. It is only fitting then, that there is increasing research on tef's phenolic profile as the need for greater inclusion of whole grain in the diet grows.

2.2 Cereal Polyphenols

2.2.1 Structure and Function of Cereal Polyphenols

Most plant polyphenols are synthesized via the phenylpropanoid biosynthesis pathway (figure 2.1).

Figure 2.1 General phenylpropanoid synthesis pathway and phenolic compound classifications.



Synthesis begins with the enzymatic conversion of phenylalanine to cinnamic acid by phenylalanine ammonia lyase (PAL), then enzyme-mediated pathways produce different classes of compounds including phenolic acids and flavonoids (Styles and Ceska 1977; Yang et al. 2016). Phenolic acids and flavonoids are concentrated in the pericarp layer of grain and can exist as free compounds, bound to sugars and soluble fiber (conjugated), or bound to insoluble fiber (bound) (Dykes and Rooney 2007; Panato et al. 2017; Ndolo, Beta, and Fulcher 2013).

Table 2.1: Examples of phenolic acids and flavonoids in cereal grains.

PHENOLIC ACIDS	Source	Reference
Hydroxycinnamic Acids (i.e. cinnamic, chlorogenic, ferulic) **Hydroxybenzoic Acids** (i.e. gallic, syringic, protocatchuic)	Wheat, purple wheat, yellow corn, oats, barley, purple barley, red rice, black rice, millet, tef, sorghum	(Subba Rao and Muralikrishna 2002; Chandrasekara and Shahidi 2011; Guo and Beta 2013; Kotaskova et al. 2016; Sumczynski et al. 2016)
FLAVONOIDS		
Flavones (i.e. apigenin, luteolin) **Flavonols** (i.e. quercetin, kaempferol) **Flavanols** (i.e. catechin) **Flavanones** (i.e. naringenin) **Various glucosides** (i.e. rutin)	Sorghum, tef, wheat, millet, purple corn	(Asenstorfer, Wang, and Mares 2006; Chandrasekara and Shahidi 2011; Ramos-Escudero et al. 2012; Gunenc et al. 2015; Moraes et al. 2015; Kotaskova et al. 2016)
Anthocyanins (i.e. cyanidin-3-O-glucoside) **Anthocyanidins** (i.e. luteolinidin, apigeninidin) **Proanthocyanidins/Tannins**	Black rice, purple rice, red rice, purple corn, sorghum	(Awika, Rooney, and Waniska 2004; Yang and Zhai 2010; Shao et al. 2014; Chen, McClung, and Bergman 2016; de Oliveira et al. 2017)

2.2.2 Factors Affecting Polyphenol Profile and Bioavailability

Pre-harvest

Since phenolic compounds are synthesized by a series of enzymatic reactions regulated by gene-expression of each respective plant there are many factors that affect variability of phenolic compounds in cereals. For example, different varieties of grains of the same species have different phenolic profiles. This is most obvious for pigmented cereals like red and purple

rice; white, red, and brown quinoa; and black or red sorghum, where profile differences are elucidated via levels and types of pigmented phytochemicals such as anthocyanidins, betalains, and anthocyanins (Awika, Rooney, and Waniska 2004, Tang et al. 2015; Chen, McClung, and Bergman 2016).

Growing conditions, both environmental and agricultural, also impact the phenolic profile of grains. Application of fertilizer and use of irrigation methods results in higher total phenolic content of winter wheat than that of grains grown with no irrigation even while adding fertilizer (Ma et al. 2014). Interestingly, in this study, the wheat was grown in two different locations, and the treatments had varied effects between locations, highlighting that location also plays a role. Phenolic compounds are synthesized as a plant's response to elements like radiation (sunlight), stress (drought/excess water), and nutrient availability (fertilizer/soil composition). Since location can affect these elements, it makes sense that it becomes a variable in understanding phenolic profiles in plants such as cereal crops.

Post-harvest Processing

Post-harvest processing such as dehulling, decortication, malting, boiling, roasting, and fermentation can result in chemical/physical changes to the phenolic profile and further influence potential bioavailability upon consumption (Taylor and Duodu 2015). Dehulling millet and decorticating sorghum decreases the total phenolic content of flours made from these processed grains (Chandrasekara, Naczk, and Shahidi 2012; Moraes et al. 2015). While cooking of millets after de-hulling also decreased the phenolic content of some species, the effect was not as pronounced across all samples as that of de-hulling (Chandrasekara, Naczk, and Shahidi 2012). As grain hulls and brans are known to contain high concentrations of phenolic compounds, illustrated by the high phenolic content values associated with concentrated hulls/brans in these

studies, their removal significantly alters the phenolic compounds that would be consumed. Here lies one potential benefit to whole grain consumption where the seed coat and bran remain in the flour used to produce foods and beverages.

Another processing method that affects the phenolic profile of cereals is fermentation, which involves using yeasts and/or lactic acid bacteria (LAB) to make breads and fermented beverages like beer (Taylor and Duodu 2015). In one study, sour dough fermentation of barley and oat groats with LAB bacteria resulted in extraction of more free, and in some cases bound, phenolic compounds than from unfermented cereals (Hole et al. 2012). Similarly, Coghe, Benoot, and Delvaux (2004) found that ferulic acid is released from barley and wheat malt during fermentation with yeast in the brewing process.

Bacterial and yeast enzymes such as feruloyl esterase are able to cleave the ester bonds of bound phenolics and release them, making them extractable in the free fraction (Coghe et al. 2004; Esteban-Torres et al. 2013). Additionally, degradation of starch and fibers by bacterial enzymes can also increase extraction of bound phenolics as they may become more accessible or more soluble and, therefore, more easily extractable after fermentation. For example, a study of the total phenolic content of fermented tef *injera* found that after 72 hours of fermentation there was a two-fold increase in extractable free and bound phenolic compounds for the four different varieties studied (Shumoy et al. 2017). Another study on *injera* found that there was a decrease in extractable phenolics over the course of fermentation, with the raw flour containing the highest level of free total phenolics (Boka, Woldegiorgis, and Haki 2013). However, the latter study discarded the *irsho* layer, a yellow liquid that forms during *injera* fermentation, before the 72 hours samples were taken, and they acknowledge that this could have removed water-soluble phenolic compounds (Boka, Woldegiorgis, and Haki 2013).

Bioavailability

The quantity of phenolic compounds metabolized by the body, and any subsequent effect on human health, is limited by the bioavailability of these compounds upon consumption. In a randomized study of 15 healthy human subjects, ingestion of wheat bread fortified with bioprocessed rye bran (treated with hydrolytic enzymes like ferulic acid esterase and fermented with yeast) resulted in 2.5 times higher ferulic acid excretion in urine than from wheat bread fortified with regular rye bran, suggesting that the higher free ferulic acid content of the bioprocessed bran resulted in higher bioavailability (Lappi et al. 2013). Only about 1% of ferulic and sinapic acid from the two rye fortified test breads was detected in the urine, and there was no detectable increase in excretion of expected ferulic and sinapic metabolites. The authors suggest that the unaccounted for ferulic and sinapic acid may not have been absorbed or that the 24 h period of data collection was not enough to capture extended circulation. However, across increasing treatment levels of total ferulic acid consumed, subjects excreted more ferulic acid in the urine as their initial intake of ferulic acid increased.

In an *in-vitro* study, Van Rymenant et al. (2017) incubated Caco-2 cells with various short chain fatty acids (SCFA; meant to mimic environment in gut after fermentation of fiber) and analyzed the transport and metabolism of ferulic acid, hesperetin (derivative of eridictyol), and salicylic acid in the cells. They found that propionate and butyrate increased the uptake and secondary metabolism of these compounds in Caco-2 cells, which may suggest a model for the role of dietary fiber on metabolism of phenolic compounds (Van Rymenant et al. 2017). Due to the complexity of both food and human metabolism, continued research should explore the fate of phenolic compounds upon human consumption and how elements like SCFA-facilitated transport of phenolics and phenolic profile of foods (free, conjugated, bound) translate to metabolic effects in the body. Therefore, it is of value to elucidate the phenolic profiles of whole grains, such as

tef, that contribute both fiber and phenolic acids in different forms, to establish a foundation for future research on digestion and biological impacts.

2.2.3 Potential Health Benefits of Polyphenols in Cereal Grains

Metabolism and Bioactivity

The potential influence of phenolic compounds, as well as bran or whole grain samples, on signaling pathways or metabolite generation for human health have been evaluated in numerous *in vivo* animal studies, *in vitro* models, and human studies. Metabolites of quercetin, chlorogenic acid, and caffeic acid, when applied to human colon cells *in vitro*, are found to down regulate gene expression of the phase I enzyme COX-2 responsible for proinflammatory response and up regulate the gene expression of phase II enzyme glutathione s-transferase which facilitates transport of toxins from the body (Miene, Weise, and Glei 2011). Pig diets high in wheat and oat bran caused effects suggesting these phenol-rich brans protect against the inflammatory response caused by a high fat diet (Rezar et al. 2003). A decrease in secretion of the pro-inflammatory marker TNF-alpha (in the presence of lipopolysaccharide stimulant) was observed in human macrophage cultures exposed to artificially digested wheat dialysate (Mateo Anson et al. 2010) and mouse leukocytes (macrophages and lymphocytes) collected and cultured after consumption of cereal supplemented diets (Álvarez et al. 2006).

Regulating the Gut Microbiota

Phenolic compounds and fiber have also been shown to alter the gut microbiota composition. A rat feeding study comparing the effect of diets containing refined rye vs. whole

rye, which was higher in phenolic and fiber content, resulted in higher levels of DHA and EPA in the blood plasma. Furthermore, whole rye fed rats exhibited a lower *Firmicute*-to-*Bacteriodetes* ratio in the gut microbiota over the 12 week study, which is sometimes associated with prevention of metabolic disorders (Ounnas et al. 2016). No difference in fiber metabolites was observed in a study in human subjects who consumed either whole grain or wheat bran cereals, though there was an observed increase in Bifidobacterium spp. over the course of the 3-week study for both diets, with a larger increase seen for the whole-grain diets. Plasma ferulic acid levels also increased for subjects on both diets that the authors attributed to the absorption of free ferulic acid and that released in the colon by the action of bacteria (Costabile et al. 2008). These studies do not make an indisputable connection between phenolic acids/fiber consumption and specific metabolic effects. However, they do illustrate the influences of these dietary components on baseline gut microbial composition and plasma metabolites, strongly suggesting that dietary phenolic compounds and/or fiber are metabolized and have the potential to influence health status.

Impacts on Digestibility

There is evidence that phenolic compounds, such as proanthocyanindins, can affect starch digestibility and therefore potentially mediate glycemic response (Amoako and Awika 2016). Phenolic extracts from grains like black rice and sorghum inhibit alpha-amylase activity resulting in less digestion of starch and less release of free glucose, while extracts from finger millet do not exhibit this inhibition (Kim, Hyun, and Kim 2011; An et al. 2016). It is likely that the differences in phenolic content between sorghum, black rice, and finger millet are responsible for the difference in effect on amylase activity.

2.2.4 Current Research on Tef Polyphenols

Research on tef polyphenols is still in its early stages. Most research on tef to date has been in the genomics field and concentrated on research to improve tef breeding, especially with respect to grain yield and grain color quality, with a preference for white grain (Assefa, Chanyalew, and Tadele 2013; Cheng et al. 2015). However, in the past decade there has been increasing interest in tef phenolics, beginning with antioxidant potential and progressing toward in-depth analyses of phenolic profiles (El-alfy, Ezzat, and Sleem 2012; Forsido, Rupasinghe, and Astatkie 2013; Salawu, Bester, and Duodu 2014; Kotaskova et al. 2016; Shumoy and Raes 2016).

Trends and Discrepancies in Tef Phenolic Research

Most studies of the phenolic profile or total phenolic content of tef offer a comparison between white and brown tef samples. While this kind of comparison can provide useful information about tef's phenolic profile, it is limited because growing conditions are often uncontrolled and unreported. Most studies use grain samples of unknown variety purchased commercially from markets as either whole grains or as whole grain flour. Shumoy et al. (2016) obtained identified varieties grown under similar growing conditions in the same growing season from the Axum Agricultural Research center in Tigray, Ethiopia. However, it is still unclear whether the samples were grown in the same location, despite being subject to similar agricultural conditions.

Furthermore, based on most cereal phenolic research, it is expected that tef would have higher bound phenolic content than free phenolic content (measured as mg gallic acid equivalents (GAE)/g sample). However, in this respect the extant literature is not consistent as Salawu et al. (2014) and Kostakova et al. (2016) reported higher free phenolic content, while Shumoy et al.

(2016) reported higher bound phenolic content. The discrepancy likely arises from the differences in extraction methodology, as different solvent mixtures, hydrolysis methods, and pH conditions were used across the three studies. Table 2.2 gives a general overview of the most abundant phenolic compounds identified in both the free and bound fractions of brown and white tef varieties reported in these three studies. While all three report ferulic acid as the most abundant bound phenolic compound, the differences reported for the free fraction is again likely due to differences in extraction methodology, but also potentially due to other factors such as variety, which remains unknown for most samples.

Table 2.2: Most abundant extractable phenolic compounds for the free and bound fractions of white and brown tef samples in three different studies (Salawu, Bester, and Duodu 2014; Kotaskova et al. 2016; Shumoy and Raes 2016).

Author	White		Brown	
	Free	Bound	Free	Bound
Salawu, Bester & Duodu	p-Hydroxybenzoic	Ferulic	p-Hydroxybenzoic Quercetin	Ferulic
Kotaskova et al.	Rutin	Ferulic	Ferulic Protocatechuic	Ferulic
Shumoy & Raes	Catechin	Ferulic Rosmarinic	Catechin	Ferulic Rosmarinic

Questions Regarding Tef Pigmentation

The identity of compounds responsible for the brown/red and white/yellow pigmentation in tef have not been confirmed. A study on red (brown) tef in Egypt identified seven ethanol extractable compounds, of which four were orange or yellow flavonoid derivatives of naringenin, eridictyol, and quercetin (El-alfy, Ezzat, and Sleem 2012). However, no comparison was done to confirm if these compounds are at all present in white tef varieties. Kotaskova et al. (2016) hypothesized that higher antioxidant activity of brown tef is correlated with its pigmentation, as is the case for

sorghum and rice, but on the other hand Salawu et al. (2014) found no significant difference in total phenolic content between the brown and white tef studied therein. Other investigators hypothesize more specifically that the color difference is related to anthocyanin content or presence of tannins in the brown tef grain (Parker, Umeta, and Faulks 1989; Umeta and Parker 1996; Shumoy and Raes 2016). Bultosa and Taylor (2004) contend that they did not detect any tannins in any of the white or brown tef samples they studied (methods and varieties not disclosed). Various physical analyses and investigations of the tef seed and seed coat suggest a fluorescent or dye-absorbing layer underneath the pericarp but it is unclear exactly what varieties were analyzed (Parker, Umeta, and Faulks 1989; Helbing 2009 as cited in Gebremariam, Zarnkow, and Becker 2012). It is reported that the seed coat of white varieties is made up of an inner integument and nucellar epidermis consisting mostly of pectin-cellulose walls covered by pericarp cells, which contain a pectin layer that forms a mucilage coating inside the cells when they are hydrated, however there was no analysis of brown seeds for comparison (Kreitschitz 2009).

2.3 Analytical Techniques for Quantifying Cereal Polyphenols and Identifying Pigments

2.3.1 Polyphenol Extraction Methods

Phenolic compounds are extracted with various methods that combine the functionality of solvents such as methanol, ethanol, acetone, diethyl ether, and ethyl acetate (Stalikas 2007). Free phenolics are extracted with pure, acidified, or mixed (aqueous) polar solvents. Methanol and acetone are usually the most efficient solvents, though acidification and mixing with water can improve extractability (Chethan and Malleshi 2007 Bangoura, Nsor-Atindana, and Ming 2013; Upadhyay et al. 2015).

Bound phenolics are most commonly hydrolyzed with 2 – 4 M sodium hydroxide for various lengths of time, usually 4 hours, but some investigators have hydrolyzed for as short as 30 min or long as 16 hours (Saulnier et al. 1999; Mateo Anson et al. 2009; Çelik, Gökmen, and Fogliano 2013; Ma et al. 2014; Shumoy et al. 2017). Kim et al. (2006) showed that hydrolysis (2 M NaOH, 4 h) resulted in more extractable phenolic acids than acidification (6 M HCl, 1 h), which can be attributed to either providing a better hydrolysis or less degradation. However, it is unclear whether the shorter time with stronger acid makes the two methods equivalent. There is always potential for either degradation due to strong acid or base, or long time hydrolysis—though these differences in hydrolysis method may also result in different compounds being extracted (Kim et al. 2006). Therefore, as with each step in an extraction, a chosen hydrolysis method should be consistent across samples to allow for comparative release of bound phenolics or multiple hydrolysis methods should be employed to observe variation in compounds released.

Most investigators report only 'free' and bound phenolic fractions. These 'free' fractions can be further divided into free phenolics and those conjugated to simple sugars or soluble fiber. Methods to separate free (relatively less water soluble) and conjugated (relatively more water soluble) phenolics start with an extraction into a polar-organic:water mixture (~80:20). Then, taking advantage of the immiscibility of less polar solvents like ethyl acetate or diethyl ether with water, and acidifying the aqueous layer to pH 2, protonated unconjugated phenolics are extracted into the organic solvent (Kim et al. 2006; Chandrasekara and Shahidi 2011; Nicoletti et al. 2013; Shao et al. 2014). After separating the organic layer from the aqueous portion, the conjugated phenolic compounds are hydrolyzed from saccharides for subsequent extraction with immiscible solvent (Nicoletti et al. 2013; Shao et al. 2014). Chandrasekara and Shahidi (2011) added an additional acid hydrolysis after this step, to hydrolyze etherified glycosidic bonds.

2.3.2 Folin-Ciocalteu Assay

The Folin-Ciocalteu assay is used to analyze the phenolic content of plant extracts. Due to the complex phenolic profiles of such extracts, it is a useful and reproducible method for relative comparison of total phenolics in a sample (Singleton and Rossi 1965). Because it is not limited to detecting phenolic compounds, most applications are used in conjunction with more specific characterization methods such as HPLC.

The active components of the Folin reagent are heteropoly acids consisting of phosphotungstate and molybdate metal complexes that oxidize the phenolates present under the alkaline conditions of the reaction (Singleton, Orthofer, and Lamuela-Raventos 1998). This reduction results in a color change of solution from yellow to blue as the phenolic compounds are oxidized. The color change is measured at 765 nm and results are compared to a standard curve of a stable phenolic compound like gallic acid to establish total phenolic content (Singleton and Rossi 1965). While Singleton and Rossi's method establishes the use of UV-spectrophotometers and cuvettes to analyze individual samples the method has been applied using 96-well plates and smaller reaction volumes to accommodate large sample sets and ease of replication of the assay (Ainsworth and Gillespie 2007; Herald, Gadgil, and Tilley 2012).

Major limitations of the Folin-Ciocalteu assay are related to overestimation of phenolic activity as ascorbic acid, fructose, iron (II), amino acids, proteins, and, in general, non-phenolic reducing groups can induce a positive response and interfere with the analysis of phenolic content (Singleton and Rossi 1965; Box 1983). Where applicable it is possible to correct for these interferences by running a Folin-Ciocalteu assay on potential interfering substances separately and then adjusting values accordingly.

2.3.3 High Performance Liquid Chromatography and Mass Spectrometry

High performance liquid chromatography is the most common approach to identifying and quantifying phenolic compounds in grains, however there is no standard method. Usually, a reverse-phase, C-18 column is used with a gradient of solvents (often times acidified with 0.1% acid) increasing from more aqueous to more organic (methanol or acetonitrile) (Guo and Beta 2013; Kim et al. 2006). Solvent mixtures can be used with gradient or isocratic elution and methods vary greatly between studies. Rao and Muralikrishna (2002) used isocratic elution with water:acetic acid:methanol (80:5:15), for an undisclosed time, to study free and bound phenolics in finger millet. Tang et al. (2015) applied a steep gradient, of increasing methanol:acetonitrile phase B from 0 to 80% in 40 minutes, and 80 -100% in 2 minutes, followed by an isocratic step for 2 min at 100%. Alternatively, Guo and Beta (2013) applied a gentle gradient, achieving an increase in mobile phase B from 9% to 70% with incremental gradients over the course of 70 minutes.

Along with solvent choice and gradient parameters, choices like column type (chemistry, diameter, particle size) also influence separation. In general, optimizing resolution of peaks requires minimization of peak broadening. Peak broadness relates to how much time the analyte spends diffusing through the pores of the column packing (Dolan 2010), which means that pore size impacts separation (though it is not commonly mentioned in phenolic extraction studies). This diffusion time is also related, albeit indirectly, to the volumetric flow rate (mL/min; set by the operator), as it is really the linear velocity, or the distance the mobile phase moves in a unit time (mm/minute) that needs to be optimized to affect diffusion time. In packed columns, linear velocity (υ_0) is determined by the relationship between volumetric flow rate (F_c), column radius (r_c), and the space available between packing particles for the mobile phase to flow (ε_υ), as seen in equation 2.1 (Poole 2003).

$$v_0 = \frac{F_c}{\pi r_c^2 \varepsilon_v} \qquad \text{(Equation 2.1)}$$

Therefore, column diameter and particle size (that determines space between particles) can also have an effect on diffusion and resolution.

Column stationary phase, or the column chemistry, affects the selectivity of the column for certain analytes. C-18 columns are packed with silica particles whose surface is modified with 18 carbon chains. This packing is relatively hydrophobic compared to the mobile phase and therefore results in longer retention of hydrophobic compounds than those that are more hydrophilic. There are a wide variety of column chemistries available, but C18 is usually the most common due to its versatility, though it may not always be the optimal choice. In any case, investigators focus on adjusting other parameters to optimize separation methods, which results in much variation. Robbins and Bean (2004), who tested various phenolic acid separation parameters, selected acidified (formic acid) solvent, a water/methanol solvent set, a combined gradient and isocratic solvent method, and a 0.7 mL/min flow rate for separation of their target phenolic acids. After testing C18 and one C8 column with various diameters, lengths, and particle sizes, and a phenyl-substituted stationary phase (a column chemistry more selective for phenolic chemical structure) a C-18 column with 150 mm length x 4.6 mm diameter; 5 µm particle size was chosen, as it had better resolution of the phenolic acid test solution, when compared to the phenyl substituted column and a column of similar length but with a 3 µm particle size.

The above studies employ UV-Vis diode array detectors (DAD) and mass spectrometers (MS) for detection and identification of phenolic compounds. With DAD detection, external standards, and sometimes an internal standard, are used for identification and quantification by creating calibration curves and comparing retention times of standards to those observed for samples (Subba Rao and Muralikrishna 2002; Robbins and Bean 2004; Kim et al. 2006). The

most common wavelengths for identification are ~280 nm and ~325 nm, as those wavelengths capture peaks for many of the common phenolic compounds, though they are not necessarily the UV-Vis peak maxima (Stalikas 2007). It is also possible with some systems to apply wavelength switching, but most studies do not use this method (Zhang et al. 2013). Mass spectral identification is usually putative, based on running standards and also matching of fragmentation patterns with comparison to available literature sources or spectral databases (Chandrasekara and Shahidi 2010; Guo and Beta 2013). It is also possible to run the MS with targeted detection, where it only detects pre-set mass transitions, but it is much more common to detect with a quadrupole time-of-flight analyzer to capture both expected and unknown compounds (Gangopadhyay et al. 2016). Some studies, like Tang et al. (2015) use both HPLC-UV-Vis (for quantification) and MS for secondary identification.

2.3.4 Principal Component Analysis

Most studies mentioned in the previous section identified compounds with external standards and/or mass-spectra and quantified them by peak area. Tabulated data provides the basis for discussion of similarities and differences with statistical models such as analysis of variance (ANOVA). This approach often limits analysis to a handful of identified peaks, especially when mass-spectra aren't available for more extensive identification. Also, it becomes more practical to point out large trends or differences, as one-to-one comparison between more than a few samples can become tedious. Principal Component Analysis (PCA) offers a different approach. PCA is a multivariate statistical method that is used to analyze data sets with many variables. The method condenses multidimensional data into fewer dimensions (principal components), representing the greatest variation in the samples (Wehrens 2011). This makes the responses/variables most responsible for the variation between samples more salient and allows

for clearer conceptualization or visualization of complex results. For example, PCA can be applied to HPLC/GC chromatograms or mass spectral data to differentiate between grains such as high-rutin and regular buckwheat or waxy and non-waxy barley, as their phenolic 'fingerprints' are unique enough to cause these varieties to group separately (Gómez-Caravaca et al. 2014; Li et al. 2014). It is also a useful approach when external standards are limited, as it allows for unknown peaks to contribute to the analysis provided that peaks can be properly aligned between samples. Like any statistical method, conclusions drawn from PCA are limited and affected by data quality and preprocessing methods.

2.3.5 Pigmentation Assays

Tannin test

A qualitative tannin test was initially developed as a quick method to determine if sorghums contain tannin or not. This test relies on stirring grain seeds with a mixture of bleach and potassium hydroxide at an elevated temperature (60 C) to remove the pericarp and reveal if the testa is pigmented. Seeds with pigmented testa will appear black and those without a testa are white/yellow. The assumption is that a pigmented testa indicates presence of tannins (Hugo and Rooney 1992).

Detection of Flavan-4-ols and 3-Deoxyanthocyanidins

Flavan-4-ols are thought to be precursors to 3-deoxyanthocyanidins. For grains like sorghum and maize, extraction of flavan-4-ols with cold methanol followed by addition of concentrated mineral acid results in a purple colored solution that can be detected at 550 nm

(Grotewold et al. 1988, personal communication with Dr. Iffa Gaffoor and Dr. Surinder Chopra 2016). This is a quick, qualitative method to determine if flavan-4-ols are present, as they can suggest presence of 3-deoxyanthocyanidins.

In the case that 3-deoxyanthocyandins are polymerized in the sample, boiling with 2M HCl for 60 min can break these tannins into 3-deoxyanthocyanins and aglycones, which are then extracted, dried, and reconstituted, in acidified solvent (Bate-Smith and Rasper 1969; Harborne 1998; personal communication with Dr. Iffa Gaffoor 2017). The extract is analyzed via UV-Vis alone or in tandem with HPLC separation. Methanol extracts containing the 3-deoxyanthocyanins luteolinidin and apigeninidin have absorbance maximums in the UV-Vis at ~490 nm and ~476 nm, respectively (Awika, Rooney, and Waniska 2004) and also can be identified in HPLC against external standards.

2.4 Summary of Literature Review

The health-benefits associated with whole-grains are linked to the higher levels of fiber and phenolic compounds these grains contribute to the diet. Numerous studies have shown that phenolic compounds and fiber have the potential to mediate inflammation and markers of disease states. However, due to the complexity of food and human metabolism, concrete cause and effect is difficult to establish. Researchers hypothesize that the type of phenolic compounds (free, conjugated, bound) important to their respective bioavailability within the body. It is therefore helpful to understand specific grain phenolic profiles for future biological studies. The phenolic profile of tef, which is consumed as a whole grain, has not been as extensively studied. There is still discrepancy as to the differences in phenolic profile between varieties, though its nutritional profile suggests that tef may have positive influence on human health. Therefore, research on the phenolic compounds present in cultivated tef varieties, while controlling for growing conditions

and location, can provide valuable insight about this whole-grain, to supplement further investigations into its potential health benefits.

2.5 Hypotheses

1. Brown and white tef varieties have different phenolic profiles that allow for distinct separation by their seed coat color in principal component analyses (PCA) and multifactor analysis (MFA) of the free, conjugated, and bound fractions.
2. The major pigments in tef are the same pigments as those found in sorghums and maize.

3. Materials and Methods

3.1 Materials

Six tef varieties (DZ-409, DZ-01-974, DZ-Cr-37, DZ-01-196, DZ-Cr-384, DZ-01-99) were grown in the same test plot in Bishoftu (Debrezeit) Ethiopia (coordinates 8.7440250, 38.964943) in 2012 (figure 3.1). The seeds were obtained from the Ethiopian Agricultural Research Institute in Debrezeit, hence the designation 'DZ'. Two commercial tef samples, one ivory (white) and one brown, were purchased from TeffCo (Idaho, USA) in 2016. All eight samples are pictured in figure 3.2. Gallic acid, p-coumaric acid, protocatechuic acid, quercetin, rutin, apigenin, naringenin, rosmarinic acid, luteolin, caffeic acid, vanillic acid, trans-cinnamic acid, chlorogenic acid, hydroxybenzoic acid, sinapic acid, syringic acid, and (+/-) catechin standards, Folin-Ciocalteu reagent, and sodium carbonate were obtained from Sigma Aldrich (St. Louis, MO 63103). Ferulic acid standard was from Fluka (now Honeywell, Morris Plains, NJ 07950). Methanol (HPLC grade), ethyl acetate (ACS grade), hexane (ACS grade), sodium

hydroxide (5 M, BDH brand), hydrochloric acid (6 M, BDH brand) were purchased from VWR (Radnor, PA 19087). Concentrated sulfuric acid (95-98%) was purchased from EMD Millipore (Billerica, MA 01821).

Figure 3.1 Image of the six tef varieties grown in Debrezeit, Ethiopia (2012).

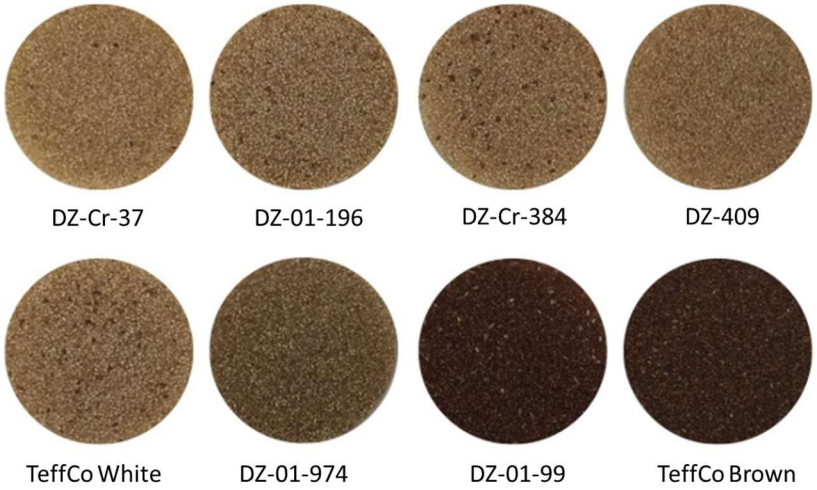


Figure 3.2 Images of the eight tef varieties analyzed in this study taken in a light box to eliminate effects of shadow and glare on seed colors.

3.2 Phenolic Extraction

Each of the eight tef grain samples was milled using an electric grain mill (Wondermill, The Wondermill Company, Pocatello ID) with the small grains adapter, on the fine (pastry) setting. The mill was cleaned between samples by milling once with white rice and twice with the next sample, all of which was discarded. Samples were randomized and milled in a different order for each replication.

All extraction procedures were done using 50 mL polypropylene centrifuge tubes as vessels. Sample flours (2 g) were defatted with hexane (5:1 v/w) for one hour with agitation on a rotary shaker (400 rpm; DS-500 Orbital Shaker, VWR, Radnor, PA). The mixture was then centrifuged (3750 rpm, 10 min, Allegra 6R Centrifuge with swinging rotor buckets and 50 mL tube inserts, Beckman Coulter Life Sciences, Indianapolis, IN) and the supernatant discarded. Defatted flour (average 1.9 g) was extracted twice with 80% methanol (5:1 v/w) under rotary shaking (400 rpm, 60 min). Samples were centrifuged after each extraction and the supernatants were decanted and combined. Flour residue was dried under vacuum in a desiccator overnight for hydrolysis the next day. Solvent was removed from the extract using a rotary evaporator (Buchi R114, Brinkman Instruments Inc., Westbury, NY) and samples were dispersed in 8 mL of 0.01 M (pH 2) hydrochloric acid (HCl). Free phenolics were extracted three times via liquid-liquid extraction with ethyl acetate (1:1, v/v) by shaking on rotary shaker (300 rpm, 15 minutes). Samples were centrifuged (3750 rpm, 10 min) between successive extractions and the supernatants were decanted and combined. Ethyl acetate extracts were dried via rotary evaporation. The aqueous portion was adjusted to pH of 14 (2 M NaOH) with 5 M sodium hydroxide and left to hydrolyze while shaking (300 rpm) for 4 h. The pH was adjusted to 2 with 6 M HCl and conjugated phenolics were extracted with ethyl acetate (1:1, v/v) following the same procedure as for the free fraction.

Dried flour residue (0.25 g) was dispersed in 6 mL of water and 4 mL of 5 M NaOH was added to result in a 2 M solution for hydrolysis. The sample was left to hydrolyze while shaking (300 rpm) for 4 h. The pH was then adjusted to 2 with 6 M HCl and bound phenolics were extracted three times with ethyl acetate (1:1, v/v). Samples were centrifuged (3750 rpm, 10 min), and the ethyl acetate layer was dried by rotary evaporation. Dried extracts were reconstituted in 80% methanol and filtered through a 0.45 μm filter before further analysis.

3.3 HPLC Analysis

An HPLC method modified from Robbins and Bean (2004) was used to identify and quantify phenolic acids and flavonoids in the tef flour extracts. The separation was conducted on an Agilent (Santa Clara, CA) 1100 series HPLC system including an online degasser (G1379A), QuatPump (G1311A), SUPELCOSIL™ C-18 reverse phase column (25cm x 4.6mm, 5um, Supelco; Bellefonte, PA), with DAD detection (G1315B) at 25 C. Ultrapure water (purified with Barnstead NANOpure Ultrapure Water System, Thermo Fisher Scientific, Waltham, MA) and HPLC grade methanol, both with 0.1% formic acid, were used as the mobile phase solvents A and B, respectively. The flow rate was 0.7 mL/min and the injection volume was 10 μL. The gradient conditions were as follows: 0 min to 10 min, 20% B isocratic; 10 min to 50 min, 30% B gradient; 50 min to 60 min, 30% B isocratic; 60 min to 85 min 70% B gradient; 85 min to 90 min, 20% B isocratic; 90 min to 95 min, 98% B gradient; 95 min to 115 min 98% B isocratic; 115 min to 120 min 20% B; gradient; 120 min to 130 min 20% B isocratic. The first 85 minutes were the analysis method, while column wash and equilibration began at 85 min to 130 min. Chromatogram signals were collected at 280 nm and 325 nm. The ChemStation (Agilent, Santa Clara, CA) UV-Vis spectrum collection feature (within method specification) was also set to store UV-Vis spectra from 200 – 900 nm in 2 nm steps. The peaks were identified by comparing

retention times and UV-Vis spectra from the DAD detector with those of external standards. Peaks were identified and quantified using HPLC-DAD data (280 nm) and external calibration curves of the standards.

Secondary, putative identification was conducted by running standards and representative samples (free (10 μL injection) and bound (10 μL injection)) of extracts from DZ-01-99 on a Shimadzu LC-10AD HPLC system (Columbia, MD) with a Waters Micromass Quattro micro API mass spectrometer, triple quadrupole mass analyzer for detection (Milford, MA). A reverse-phase Eclipse Plus C18 column (150mm x 2.1mm x 5um, Agilent, Santa Clara, CA) was used. The LC-MS was operated over a scan range of m/z 100 - 726 with a drying gas (Argon) temperature of 350 C, a drying gas flow of 650 L/h, cone gas flow rate of 50 L/h, a cone voltage of 25 V, and capillary voltage of 3000 V, and an ion source temperature of 120 C. HPLC-MS grade ultrapure water and methanol, each with 0.1% formic acid were used as mobile phase A and B, respectively. The gradient method was as follows: 0 min to 10 min 20% B isocratic; 10 min to 50 min 30% B gradient; 50 min to 60 min 30% B isocratic; 60 min to 95 min 70% B gradient; 95 min to 110 min 100% B gradient; 110 min to 115 min 100% B isocratic; 115 min to 120 min 20% B gradient; 120 min to 125 min 20% isocratic.

3.4 Folin-Ciocalteu Assay

The Folin-Ciocalteu assay was performed as described in Waterhouse (2001). Briefly, 20 μL of sample was pipetted into 1.58 mL of distilled water and 100 μL of Folin-Ciocalteu reagent was added. Samples were vortexed and, after ~6 min, 300 μL of sodium carbonate solution (200 g/L) was added and samples vortexed again. The samples were incubated at 37 C for 30 minutes. After transfer of 200 μL into a 96 well plate, absorbance was read at 765 nm (Multiskan™ GO

Microplate Spectrophotometer and SkanIt™ software, Thermo Scientific, Waltham, MA). A gallic acid standard curve was used for quantification.

3.5 Detection of Flavan-4-ols and 3-deoxyanthocyanins

This method was conducted as discussed with Dr. Iffa Gaffoor and Dr. Surinder Chopra (2016). Immature tef seeds (~100 count; ~4 mo. after planting) collected from tef (TeffCo Brown and DZ-Cr-37) grown in a greenhouse on the Penn State campus and a positive control (immature purple maize pericarp; provided by Iffa Gaffoor, Penn State Plant Science Department) were soaked in cold methanol (1 mL, 4 C) for a week. Supernatants were collected and treated with cold concentrated sulfuric acid (750 μL sample:50 μL acid, 4 C). The supernatants were subsequently analyzed via spectrophotometer (GENESYS™ 10S UV-Vis, Thermo Scientific, Waltham, MA) from 200-800 nm.

3.6 Anthocyanin Extraction

The anthocyanin extraction method was modified from Harborne (1998). Tef seeds (5 g; DZ-01-99, DZ-384, TeffCo Brown) were soaked in 10 mL of water overnight then milled with the water using a homogenizer (PowerGen 125, Fisher Scientific, Pittsburgh, PA). The suspension was adjusted to pH 2 with hydrochloric acid (12 M) and the samples were incubated for 1 h at 100 C. Samples were then centrifuged (4000 rpm; 10 min) and the supernatant was removed. Supernatants were extracted with an equal volume of isoamyl alcohol by shaking at 400 rpm for 15 min. Samples were centrifuged (4000 rpm; 10 min) and the supernatant was decanted. The extraction was done three times and the pooled supernatant was evaporated to dryness. Dry sample was reconstituted in acidified methanol (0.1% HCl) and analyzed with a

UV-Vis spectrophotometer (Spectronic Helios alpha, Thermo Fischer Scientific, Waltham, MA). Samples were also analyzed via HPLC using the method described in the appendix (personal communication with Dr. Iffa Gaffoor and Dr. Surinder Chopra 2016).

3.7 Extended Hydrolysis

Whole tef seeds of DZ-01-99 and DZ-Cr-37 (0.5 g) were hydrolyzed at room temperature with 2 M sodium hydroxide (50 mL) with samples of the supernatant (2-3 mL) being collected at 0, 1, 10, 24, and 40 hours. The 1 and 24 h supernatant samples (1.5 mL each) were transferred to Eppendorf tubes and centrifuged (3 min, 14000 rpm; Microfuge16, Beckman Coulter Life Sciences, Indianapolis, IN). Then the UV-VIS spectra of supernatants were collected with a UV-Vis spectrophotometer from 300-800 nm (GENESYS™ 10S UV-Vis, Thermo Scientific, Waltham, MA).

3.8 Tannin Test

Potassium hydroxide (7.5 g) was dissolved in a solution of water (25 mL) and bleach (8.25% sodium hypochlorite, 45 mL). Each of the tef seed varieties (0.05 g), as well as a positive and negative sorghum control, was stirred vigorously in 10 mL of this solution, at 60 C, for 10 min. Seeds were then washed with cold water, dried, and analyzed under a microscope (Nikon SMZ1000 zoom stereomicroscope with a DXM1200 Digital Still Camera, Melville, NY).

3.9 Statistical Analysis

Extractions were completed in triplicate, except for samples of bound DZ-01-196 and free DZ-409, which were completed in duplicate due to an accidental loss of sample. Folin-Ciocalteu analyses were completed in triplicate for each replicate, averaged and then the replicates were analyzed via ANOVA. For ANOVA analyses significance was established at α=0.05, and means compared using Tukey's test at α=0.05. A table of raw data with peak areas and approximate retention times (used to align peaks) is available in table A.1 of the appendix.

3.9.1 HPLC-DAD Standard Calibration and Principal Component Analysis

Integrated chromatograms of extraction replicates were aligned within each fraction based on peak retention times and UV-Vis spectra comparison (n=2 for free (DZ-409, DZ-01-974, DZ-Cr-37, DZ-Cr-384), conjugated (DZ-409), bound (DZ-01-196; n=3 all others). Missing, or 'zero', peaks were assigned a value of 0.01 multiplied by the limit of detection for the calibration curve as it could not be confirmed compounds were not present, rather that they were below the limit of detection. Peak areas were converted to μg compound/g defatted tef flour (DTF) using HPLC-DAD derived calibration curves of external standards. For peaks identified as corresponding to present standards, primarily by retention time and UV-Vis spectra, as well as secondary putative confirmation of m/z and retention time by mass spectrometry (appendix table A.2), specific calibration curves were used. The hydroxybenzoic (OH-benzoic) acid curve was used to report 'OH-benzoic acid equivalents' of unidentified peaks as this compound had the lowest limit of detection (ferulic acid equivalents were used for peak 57 in the free fraction because it was out of range of the OH-benzoic curve).

Limits of detection (LOD_C) and limits of quantification (LOQ_C) of the calibration curves were determined using the least-square method (LINEST function) in Microsoft Excel. The function was set to force the line through zero when the absolute value of the y-intercept was less than or equal to the standard error of the y-intercept (Dolan 2009). The LOD_C and LOQ_C (μg compound/mL) were evaluated as shown in equations 3.1 and 3.2 (International Committee on Harmonization 1996). The LOD and LOQ for the extraction and quantification method (μg compound/g DTF) were determined by dividing LOD_C and LOQ_C by grams of DTF extracted/mL for each fraction.

$$LOD\_C = 3.3\left(\frac{SE_y}{Slope}\right) \qquad \text{(Equation 3.1)}$$

$$LOQ\_C = 10\left(\frac{SE_y}{Slope}\right) \qquad \text{(Equation 3.2)}$$

Peak concentrations were converted to μg/g DTF and evaluated to determine those that were <LOQ or <LOD of the method (μg compound/g DTF). Detailed calibration curve parameters, LOD, and LOQ for identified compounds, and sample information can be found in tables A.3 and A.4 of the appendix.

In order to determine significant differences, peak concentrations reported as <LOD or <LOQ were converted to numerical values. Peak concentrations labeled as <LOD were assigned values of the LOD multiplied by 0.01 and peaks labeled as <LOQ were assigned values of the average of LOD and LOQ. Minitab analysis with the Kruskal-Wallis test for non-parametric data was used to determine significant differences between varieties for each peak (α=0.1, n=2 for free (DZ-409, DZ-01-974, DZ-Cr-37, DZ-Cr-384), conjugated (DZ-409), bound (DZ-01-196; n=3 all others; Minitab Inc., State College, PA). After calculating medians of peak concentration, values were evaluated again to check if any fell below LOD and LOQ and were adjusted accordingly. Values were reported as <LOD, <LOQ, or median concentration in μg/g DTF.

Significant peaks, as determined by the Kruskal-Wallis test ($p<0.01$), and significant Folin-Ciocalteu results were organized in a matrix format with response variables as columns and sample varieties as rows. The principal component and multifactor analyses were run using a correlation matrix and the SensoMineR package in RStudio (Boston, MA). Median comparisons between varieties for significant peaks where all values >LOQ were determined with the Minitab Kruskal-Wallis Multiple Comparisons macro (KRUSMC.mac).

4. Results and Discussion

4.1 Folin-Ciocalteu Assay

Folin-Ciocalteu results shown in table 4.1 reveal that the bound fraction contributes most to the total phenolic content for all varieties. Within fractions there is no significant difference between varieties for the free and bound fractions ($p>0.05$). DZ-01-974 (Dukem) and DZ-Cr-37 (Tsedey) show significantly higher phenolic content in the conjugated fraction than the DZ-409 (Boset), DZ-01-196 (Magna), DZ-01-99 (Asgori, brown), TeffCo White, and TeffCo Brown, while the DZ-Cr-384 (Kuncho) shows significantly less phenolic content in the conjugated fraction. Though the differences are statistically significant in the conjugated fraction, the total phenolic concentration is lowest in this fraction. With respect to overall phenolic profile as a potential influence on digestion and metabolism, all varieties contribute similar quantities of free, conjugated, bound and total phenolics. Overall, phenolic content results are consistent with Shumoy et al. (2016) as bound fractions having higher values than free/conjugated (soluble), however they are also consistent with Salawu et al. (2014) with respect to no significant differences between the free fraction total phenolic content of brown and white tef (varieties not

disclosed). Unlike Shumoy et al. (2016) and Kotaskova et al. (2016) there are no distinct differences between white and brown varieties with respect to total phenolic content.

Table 4.1: Folin-Ciocalteau results for free, conjugated and bound fractions in mg gallic acid equivalents (GAE) per gram of defatted tef flour. Different lower case letters indicate significant difference within a fraction ($p<0.05$) and p-values are noted in parentheses next to fraction label (n=3 for all except DZ-409 Free and Conjugated, DZ-01-196 Bound where n=2).

Variety	Free (0.15)	Conjugated (0.02)	Bound (0.08)	Total (0.05)
DZ-409	0.15 ± 0.010 a	0.07 ± 0.005 ab	0.92 ± 0.118 a	1.19 ± 0.139 a
DZ-01-974	0.18 ± 0.011 a	0.08 ± 0.012 a	1.10 ± 0.019 a	1.37 ± 0.015 a
DZ-Cr-37	0.15 ± 0.018 a	0.08 ± 0.007 a	0.91 ± 0.041 a	1.14 ± 0.022 a
DZ-01-196	0.17 ± 0.027 a	0.07 ± 0.007 ab	0.80 ± 0.153 a	1.03 ± 0.111 a
DZ-Cr-384	0.19 ± 0.056 a	0.05 ± 0.003 b	1.12 ± 0.206 a	1.37 ± 0.207 a
DZ-01-99	0.17 ± 0.009 a	0.06 ± 0.004 ab	1.08 ± 0.042 a	1.31 ± 0.054 a
TeffCo White	0.12 ± 0.013 a	0.06 ± 0.011 ab	0.93 ± 0.078 a	1.12 ± 0.085 a
TeffCo Brown	0.15 ± 0.035 a	0.06 ± 0.010 ab	0.99 ± 0.152 a	1.20 ± 0.178 a

4.2 High Performance Liquid Chromatography with Diode Array Detection (HPLC-DAD) Identification and Quantification Results

HPLC chromatograms of tef extracts exhibited peaks with fluctuating retention times (± 0.5 minutes) which required peak alignment within fractions. For example, the representative chromatograms of the free fraction of three tef varieties shown in figure 4.1 share a peak at ~51 minutes. The UV-Vis spectrum of the ~51 minute peak was identical between the varieties and so the retention times of the peaks were adjusted to align them accordingly.

Most HPLC-DAD peaks across the three fractions for all eight varieties remained unidentified when compared to standards. Protocatechuic acid in the free and conjugated fractions, as well as hydroxybenzoic, vanillic, syringic, and ferulic acids in all fractions, were identified and quantified. Results are shown in table 4.2. Most of these compounds did not exhibit a significant difference in median concentrations between tef varieties as determined by the Kruskal-Willis method ($p>0.1$).

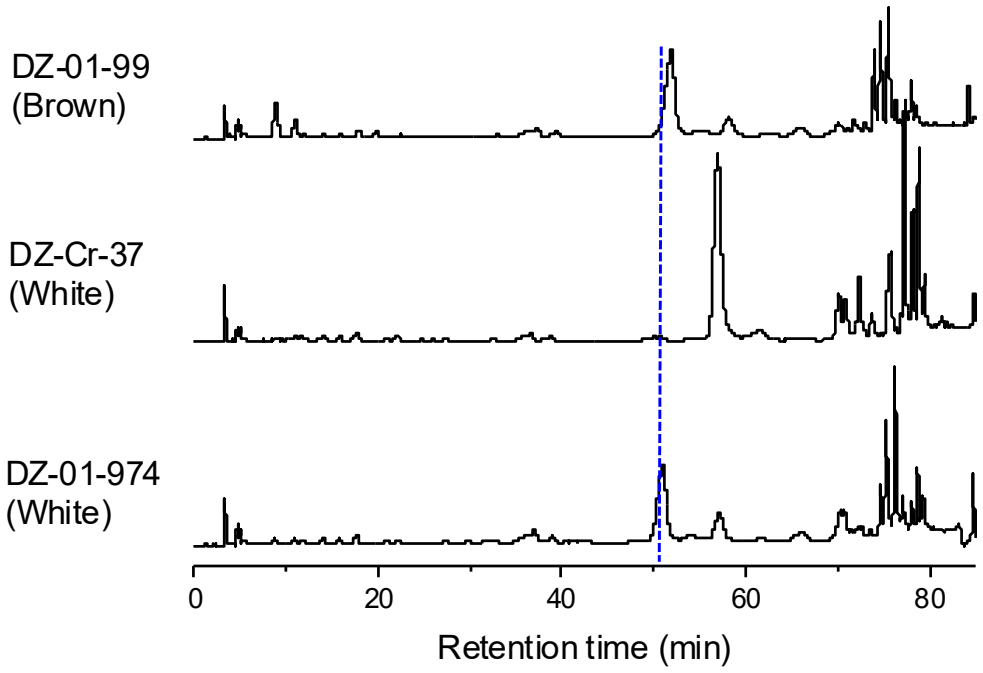


Figure 4.1 HPLC Chromatograms (280 nm) of the DZ-01-99, DZ-Cr-37, DZ-01-974 free fraction extracts and an example of peak alignment at ~51 minutes denoted by the dotted vertical line.

The exception was ferulic acid in the bound fraction, where there were significant differences in ferulic acid levels between varieties ($p<0.1$). DZ-Cr-384 exhibited the highest ferulic acid content (median value 280.73 μg/g DTF). While Kotaskova et al. (2016) and Shumoy et al. (2016) report higher levels of bound ferulic acid in some brown varieties than in white, Salawu et al. (2014) report higher cell-wall bound ferulic acid in white (394 μg/g) as compared to brown (142 μg/g). Shumoy et al. (2014) also studied the DZ-Cr-384 (Kuncho) variety and found it had the highest free ferulic acid content (24 μg/g) and second highest bound ferulic acid content (466 μg/g; after one of two brown varieties studied) when compared to other brown and white varieties. As expected, bound fractions of all varieties contained much higher levels of ferulic acid than in the conjugated or free fractions.

Table 4.2: Tabulation of identified and unidentified peaks from free (A), conjugated (B), and bound (C) fractions of eight tef varieties. Values are presented as μg compound per gram of DTF with unidentified peaks presented as OH-benzoic equivalents (A57 is given in ferulic acid equivalents). Peaks with the same retention time but different UV-Vis spectra are denoted with an underscore (i.e. 51_). Values of <LOD and <LOQ indicate tha the concentration was below limit of detection and quantification, respectively. Values are medians of extraction replications (n =2 for free (DZ-409 DZ-01-974, DZ-Cr-37, DZ-Cr-384), conjugated (DZ-409), bound (DZ-01-196); n=3 for all others). Shaded rows indicate significant difference in the medians of peak concentration between varieties as determined by the Kruskal-Wallis test (α=0.1). Significant rows where all values are >LOQ were tested using the Kruskal-Wallis multiple comparison macro in Minitab (KRUSMC.mac) and median comparisons are denoted with lowercase letters. Values within a row that have the same letter are not significantly different (family α=0.2; Bonferroni pairwise α=0.007).

	FREE FRACTION								Kruskal-Wallis Test	
Approx, RT	DZ-409	DZ-01-974	DZ-Cr-37	DZ-01-196	DZ-Cr-384	DZ-01-99	TeffCoWhite	TeffCoBrown	p-value:	p adj for ties
A5.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A9_Protocat.	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOQ	<LOD	<LOD	0.397	0.123
A10	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A11	<LOD	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	1.000	---
A12	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A14_Benzoic	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A16	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A18_Vanillic	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A20	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A22_Syrin.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A28	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A30	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A33_pCoum.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A33.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A37_Ferulic	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A39	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---

Table 4.2 continued.

	FREE FRACTION								Kruskal-Wallis Test	
Approx. RT	DZ-409	DZ-01-974	DZ-Cr-37	DZ-01-196	DZ-Cr-384	DZ-01-99	TeffCo White	TeffCo Brown	p-value:	p adj for ties
A51	<LOD	19.69	<LOD	6.06	<LOD	25.35	<LOQ	21.71	0.015	0.012
A51_	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A54	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	0.439	0.093
A54_	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A57	47.46	<LOQ	47.05	44.02	52.60	<LOD	<LOQ	<LOD	0.025	0.019
A62	<LOQ	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOQ	<LOD	0.093	0.021
A66	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOQ	0.057	0.008
A70	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	0.313	0.045
A71	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	0.087	0.024
A72.5	6.43	<LOD	<LOQ	6.90	5.77	<LOD	<LOQ	<LOD	0.046	0.03
A74	<LOQ	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD	<LOD	0.405	0.12
A74.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A75.5	<LOQ	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOQ	<LOD	0.141	0.042
A75.8	<LOQ	<LOD	<LOQ	<LOD	<LOQ	<LOD	<LOQ	<LOD	0.076	0.022
A76.4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A76.8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A77	10.45	<LOQ	10.94	5.75	9.20	<LOQ	6.48	<LOQ	0.083	0.055
A78	8.71	7.73	6.98	9.33	10.70	6.88	6.53	<LOQ	0.169	0.169
A78.8	12.66	10.84	10.69	9.36	13.89	8.32	8.90	7.25	0.202	---
A79	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOD	<LOQ	<LOD	0.26	0.102
A80.2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
A80.5	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	0.152	0.008
A81	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.989	0.253
A81.8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---

Table 4.2 continued.

	CONJUGATED FRACTION								Kruskal-Wallis Test	
Approx. RT	DZ-409	DZ-01-974	DZ-Cr-37	DZ-01-196	DZ-Cr-384	DZ-01-99	TeffCo White	TeffCo Brown	p-value:	p adj for ties
B6.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
B8_Protocat.	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOQ	0.467	0.233
B9	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.997	0.464
B11.5	<LOQ	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOQ	<LOD	0.467	0.233
B12	<LOQ	6.0075	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.788	0.603
B14_Benzoic	6.0842	6.789	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.447	0.222
B15	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.808	0.276
B16	<LOQ	<LOQ	<LOD	<LOQ	<LOQ	<LOD	<LOQ	<LOD	0.389	0.197
B18_Vanillic	14.67245	13.635	15.5563	13.92	15.015	17.3302	<LOQ	15.6423	0.172	0.162
B20.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
B22.5_Syrin.	<LOQ	12.9525	<LOQ	11.6501	<LOQ	<LOQ	<LOQ	11.799	0.483	0.201
B24	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
B30	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
B33_pCoum.	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
B36.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
B38	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.853	0.052
B39_Ferulic	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
B50	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.997	0.464
B57.5	9.9703	<LOD	9.9426	7.7924	12.3618	<LOD	<LOQ	<LOD	0.009	0.006
B70.5	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	0.817	0.15
B71	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	0.817	0.15
B72	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
B72.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
B73.5	<LOQ	<LOQ	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOD	0.129	0.036

Table 4.2 continued.

	CONJUGATED FRACTION								Kruskal-Wallis Test	
Approx. RT	DZ-409	DZ-01-974	DZ-Cr-37	DZ-01-196	DZ-Cr-384	DZ-01-99	TeffCo White	TeffCo Brown	p-value:	p adj for ties
B75	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
B76	<LOQ	<LOD	<LOQ	<LOD	<LOD	<LOD	<LOD	<LOD	0.773	0.338
B76.8	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.946	0.478
B78	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
	BOUND FRACTION								Kruskal-Wallis Test	
Approx. RT	DZ-409	DZ-01-974	DZ-Cr-37	DZ-01-196	DZ-Cr-384	DZ-01-99	TeffCo White	TeffCo Brown	p-value:	p adj for ties
C7	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.972	0.643
C14_Benzoic	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.968	0.751
C16	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C18_Vanillic	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
21	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C22_Syringic	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C30	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C33_pCoum.	<LOD	<LOD	<LOD	<LOD	<LOQ	<LOD	<LOD	<LOD	0.871	0.15
C38	54.12	74.41	62.31	51.43	79.8	61.63	72.35	55.81	0.498	0.496
C39_Ferulic	212.42 a	265.43 ab	221.53 a	235.09 ab	280.73 b	243.16 ab	243.54 ab	249.47 ab	0.05	---
C57	<LOQ	<LOD	<LOQ	<LOQ	<LOQ	<LOD	<LOQ	<LOD	0.103	0.02
C57_	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C73.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C74.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C75.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C77	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C78	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1.000	---

Table 4.2 continued.

	BOUND FRACTION								Kruskal-Wallis Test	
Approx. RT	DZ-409	DZ-01-974	DZ-Cr-37	DZ-01-196	DZ-Cr-384	DZ-01-99	TeffCo White	TeffCo Brown	p-value:	p adj for ties
C78.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C78.8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C79	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C80	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C80.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C81.4	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C82	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C82.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C83	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---
C83.5	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	1.000	---

4.3 Principal Component Analysis and MFA of Free, Conjugated, and Bound Fractions

4.3.1 Principal Component Analysis

Principal component analyses conducted on the free, conjugated, and bound fractions are shown in figures 4.2, 4.3, 4.4, respectively. The free and bound fraction PCA analyses exhibit clear separation between the brown and white varieties with the exception of the DZ-01-974 which groups with the browns. In the conjugated fraction PCA, the DZ-01-974 variety is grouped separately from the other varieties in the first principal component dimension. Visually, as seen in figure 3.2, DZ-01-974 (Dukem) is darker than most of the white varieties, and this slight difference could be why this variety groups more closely with brown varieties in most fractions. The free fraction, figure 4.2, has the greatest separation in the first and second principal component as compared to the other fractions as the x-axis spans from -4 to 4 units. This suggests that the greatest difference between clusters is occurring in the free fraction.

Loading plots show the influence of individual peaks on PCA separation. For the free fraction loading plot shown in figure 4.5, separation in the first principal component dimension (PC1) is determined by peaks with retention times over 50 minutes. Most peaks eluting between 72.5 – 77 minutes correlated with white varieties and peaks at 51 and 66 minutes correlated with brown varieties and DZ-01-974. The peak eluting at 80.5 minutes drives separation in the second principal component dimension (PC2) of DZ-01-99 and DZ-01-974 from the TeffCo Brown. Peaks at 70 and 71 minutes drive the separation in PC2 of white varieties, but the effect is not as pronounced as that seen with the browns and DZ-01-974, so there is not as strong of a separation between the TeffCo White and the other white varieties. It is difficult to determine if the large separation of the TeffCo Brown and the slight separation of TeffCo White (in the direction of

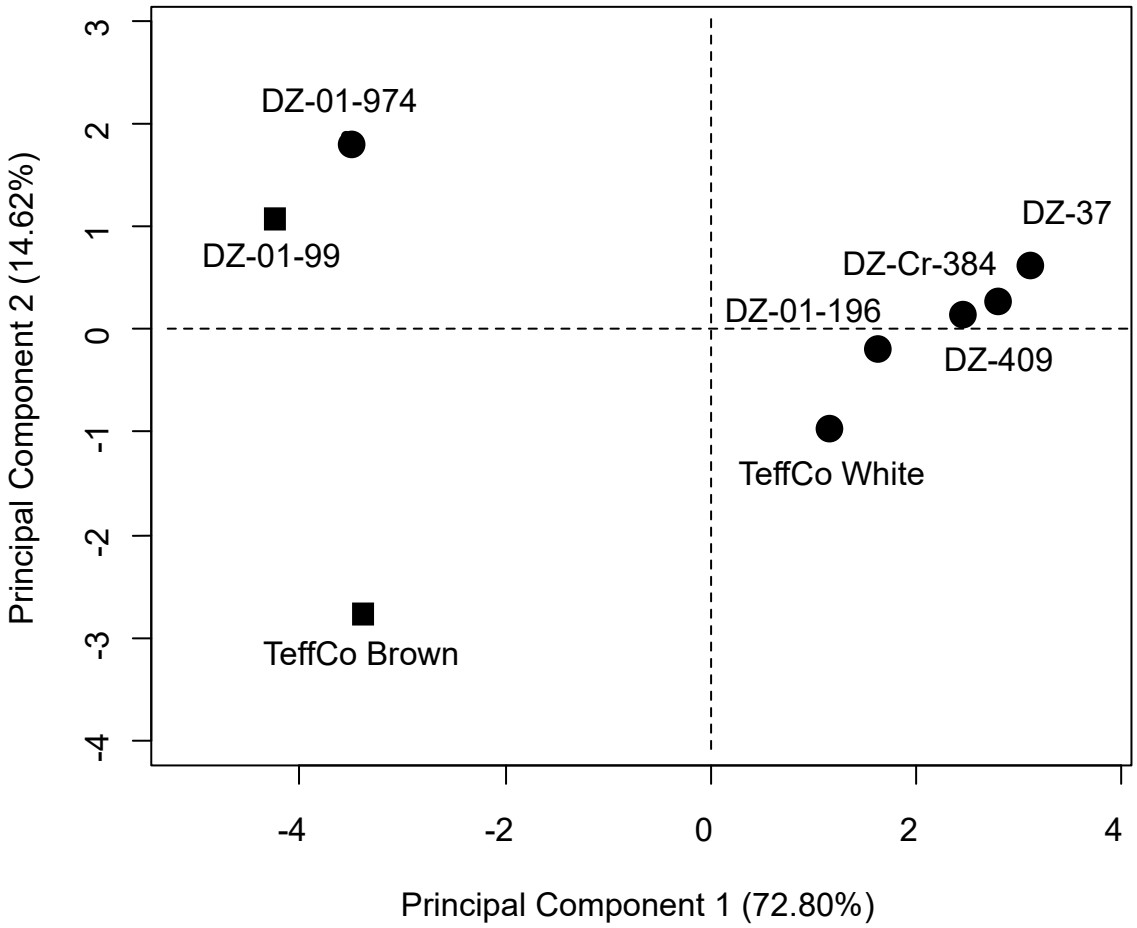


Figure 4.2 Score plot from PCA based on HPLC data for the free fraction for eight tef varieties. Brown varieties are marked with square points.

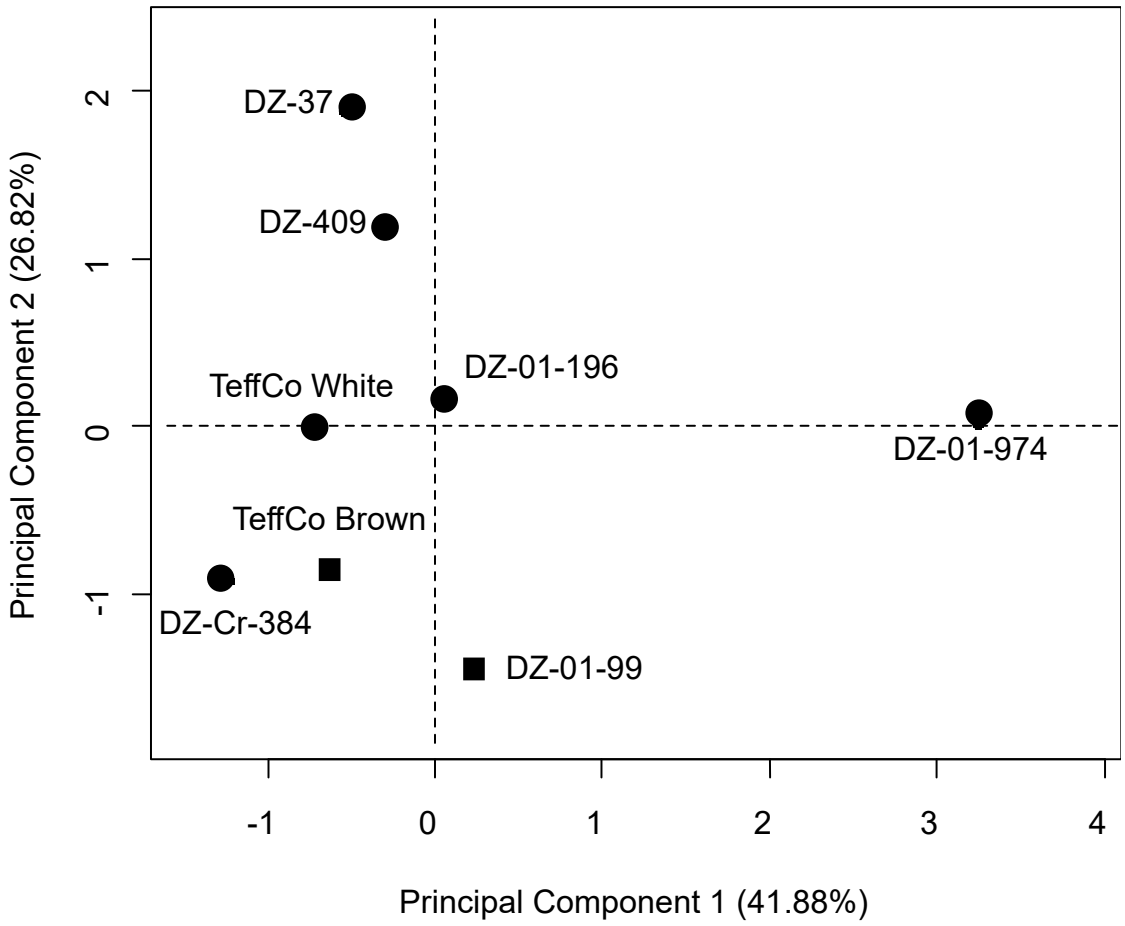


Figure 4.3 Score plot from PCA based on HPLC data and Folin-Ciocalteu results for the conjugated fraction for eight tef varieties. Brown varieties are marked with square points.

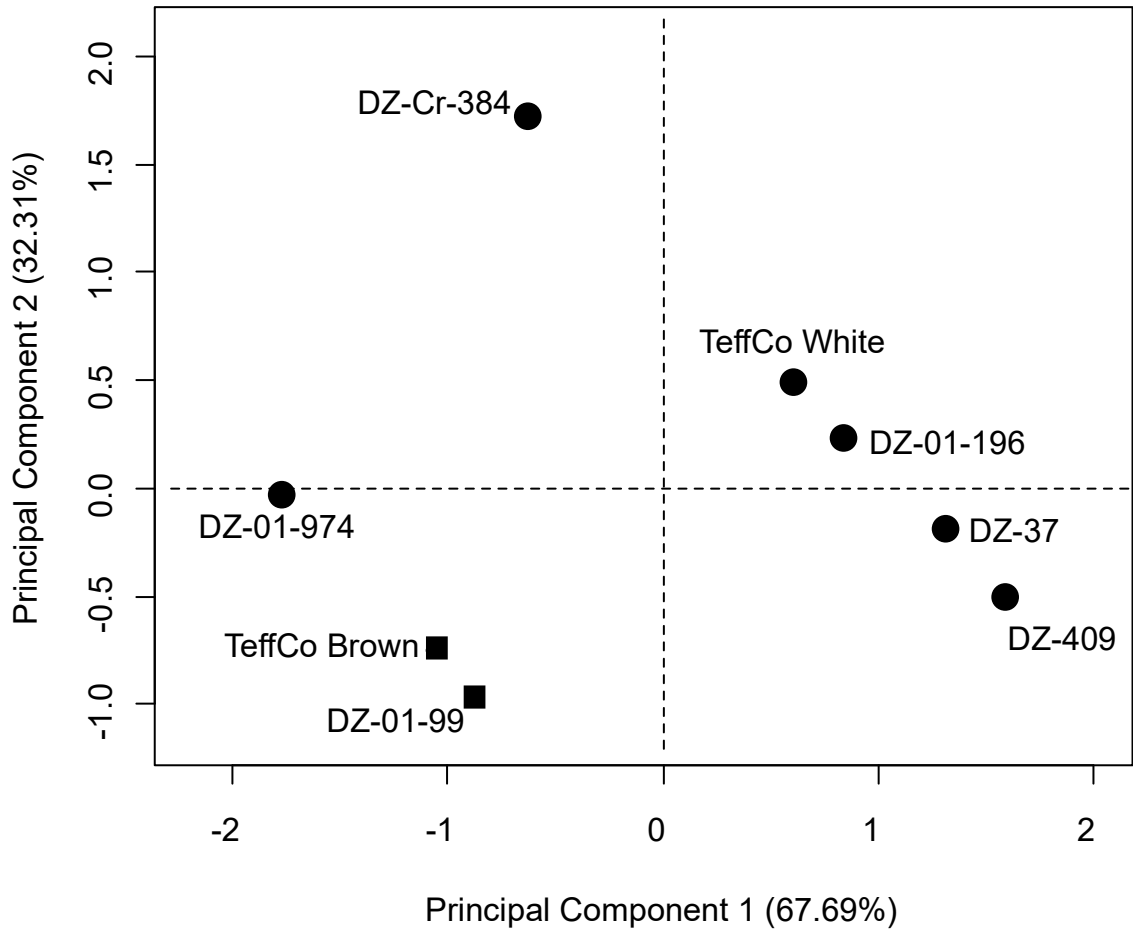


Figure 4.4 Score plot from PCA based on HPLC data for the bound fraction for eight tef varieties. Brown varieties are marked with square points.

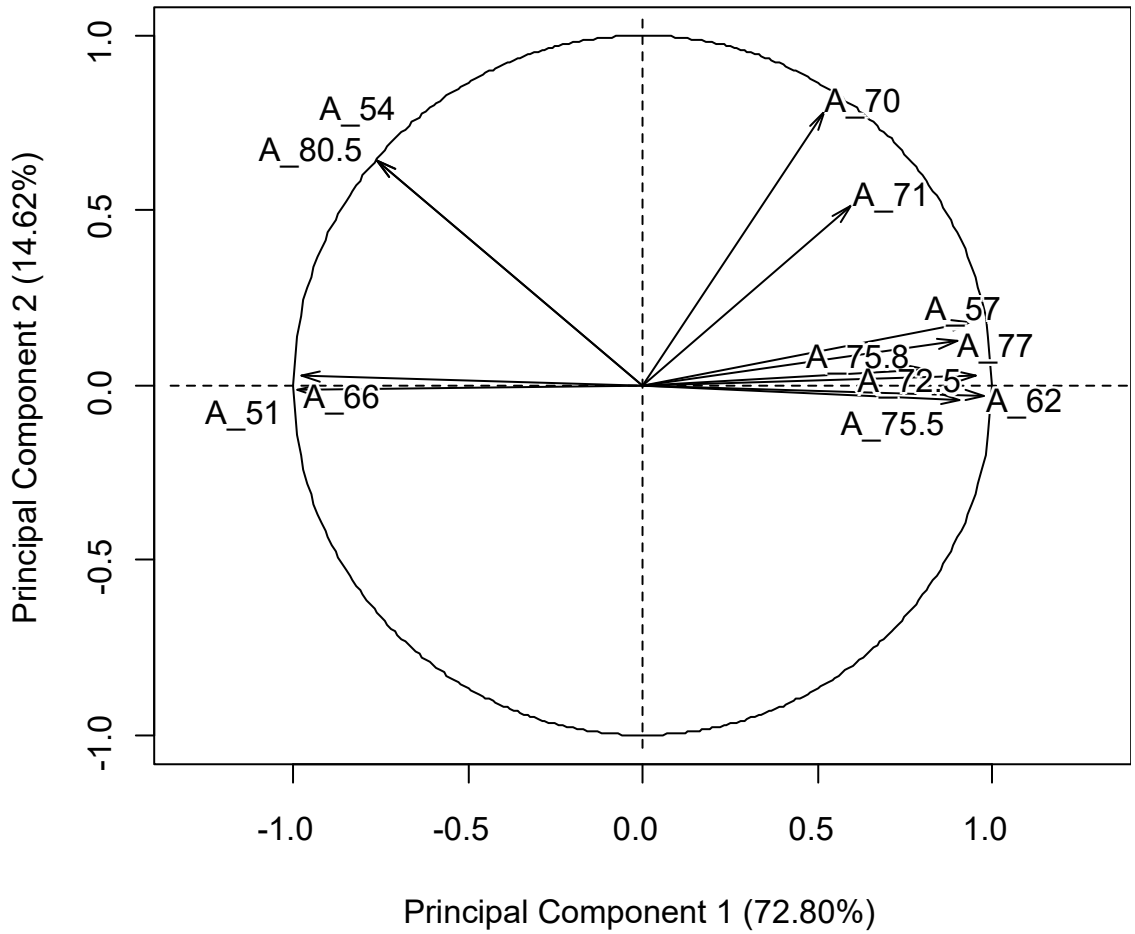


Figure 4.5 Loading plot from PCA of significant HPLC peaks in the free fraction for eight tef varieties. Identified peaks are labeled with retention time and compound name, and unidentified peaks are labeled with retention times.

TeffCo Brown) can be an effect of growing location or age of the sample (including storage time after harvest) as the varieties and ages of these samples are unknown.

With regard to the conjugated fraction, as seen in the figure 4.6 loading plot, peaks 57.5, 38 and Folin-Ciocalteu results correlate with white varieties, and are inversely correlated with the browns and DZ-Cr-384. This result also corroborates the Folin-Ciocalteau values, where the two brown varieties, TeffCo White, and DZ-Cr-384 have smaller conjugated phenolic content values than the other white varieties. Moreover, peaks 73.5 and 38 also drive separation in PC1 between the DZ-01-974 and other white varieties.

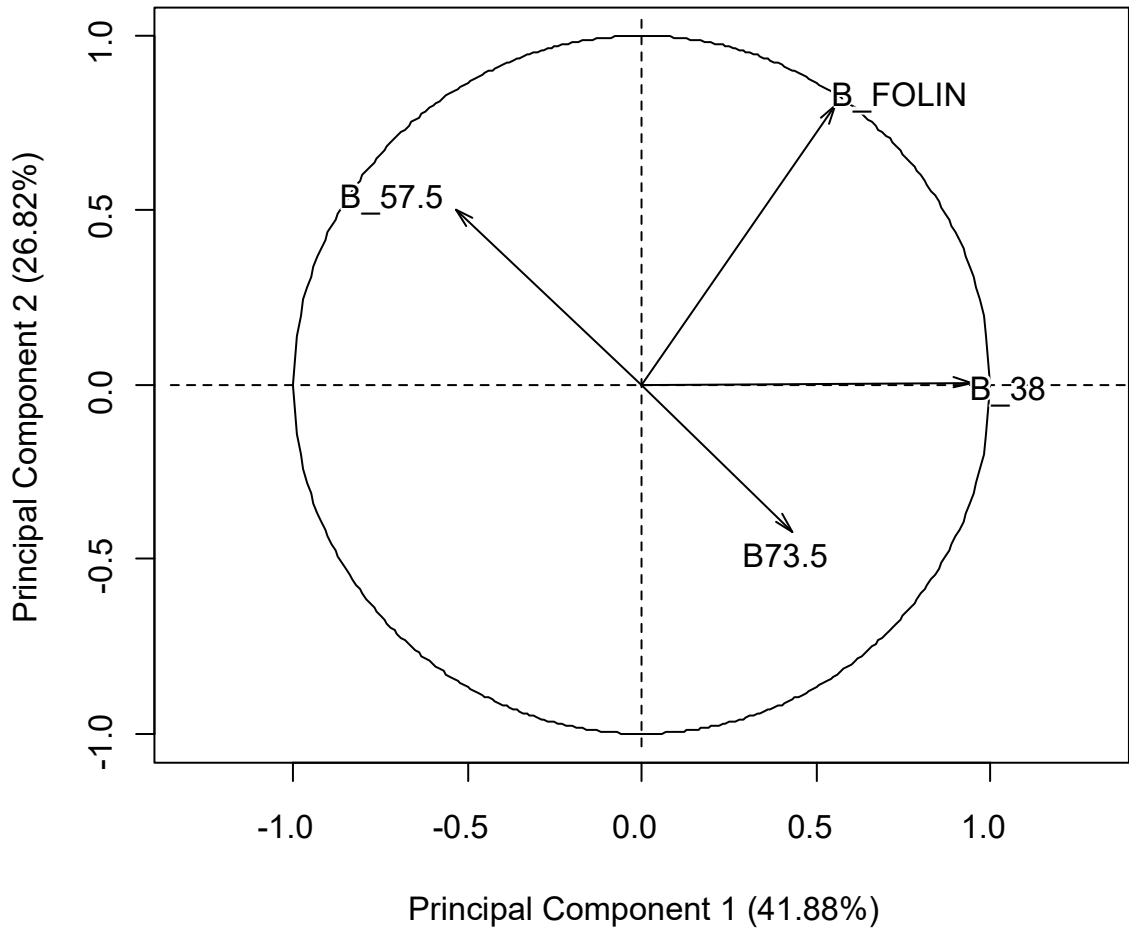


Figure 4.6 Loading plot from PCA of significant HPLC peaks and Folin-Ciocalteu assay results in the conjugated fraction for eight tef varieties. Identified peaks are labeled with compound name, and unidentified peaks are labeled with retention time.

Finally, considering the bound fraction loading plot in figure 4.7, there are two statistically significant peaks that contribute to separation between bound fractions of varieties: ferulic acid and the peak eluting at 57 minutes. Peak 57 correlates positively with white varieties in the PC1 and PC2 dimensions, while correlating negatively with the brown varieties and DZ-01-974. The presence of ferulic acid drives separation in PC2 of DZ-Cr-384 (Kuncho) from all other varieties. This is expected, as Kuncho has the highest bound ferulic acid content.

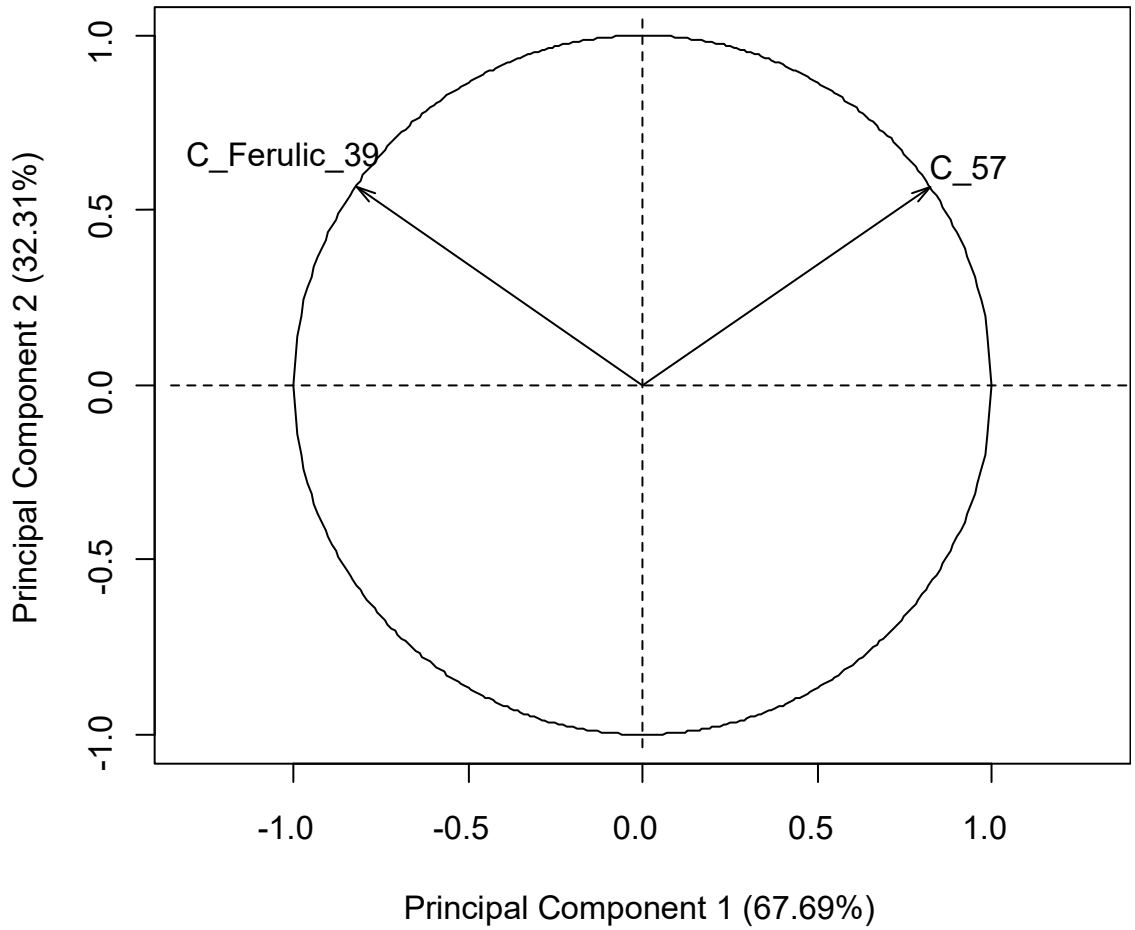


Figure 4.7 Loading plot from PCA of significant HPLC peaks in the bound fraction for eight tef varieties. Identified peaks are labeled with compound name, and unidentified peaks are labeled with retention time.

Overall, peaks eluting at later times correspond to larger and more hydrophobic compounds as illustrated by phenolic acid standards such as trans-cinnamic acid and rosmarinic acid, as well as flavonoids, which elute in the region above 45 minutes. As HPLC peak data and further PCA analysis indicate, most phenolic acids do not differ significantly between varieties, and those that are significantly different offer separation in the vertical (second) component. The largest contribution to differences between brown and white varieties comes from compounds eluting above 45 minutes, which are likely to be large phenolic acids and flavonoids. While none of the flavonoid standards could be positively identified in the HPLC chromatograms, flavonoids have been previously identified in tef by other authors (Kotaskova et al. 2016; Habtu Shumoy and

Raes 2016; El-alfy, Ezzat, and Sleem 2012). There are many flavonoid compounds and standards that have not been analyzed in tef, so it is possible that the compounds extracted from these varieties, which elute at these time points have not yet been studied.

4.3.2 Multifactor Analysis

While individual PCAs were used to compare phenolic extracts within fractions, there are also differences between the three fractions, even within one variety (figure 4.8). Unlike the PCA peak alignment within fractions, it was difficult to make definitive decisions concerning peak alignment based on retention time and UV-Vis spectra between fractions.

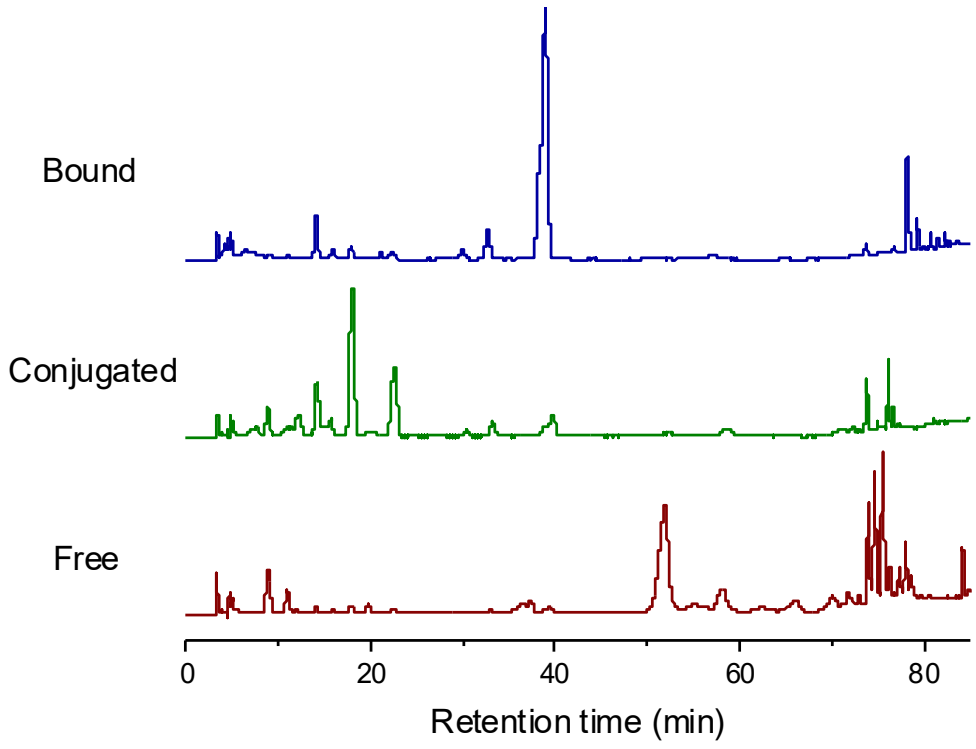


Figure 4.8 Representative HPLC chromatogram (280 nm) of the free, conjugated, and bound fractions extracted from DZ-01-99.

Therefore, an MFA was used on the free, conjugated, and bound PCA datasets. Multifactor analysis (MFA) of the three PCA data sets shows a clear separation between TeffCo Brown, DZ-01-99, DZ-01-974 and the other white varieties as seen in figure 4.9. In the MFA, free (red), conjugated (green), and bound (blue) fractions as seen in individual PCA plots are represented as extensions of the black 'composite' MFA points. For most of the varieties, the individual fraction points extend far from the composite point suggesting that all three fractions contribute to the overall differences observed between varieties.

Table 4.3 shows how each of the three fractions contributes to the MFA separation. The free, conjugated, and bound fractions contribute equally to the separation along PC1, while the conjugated and bound fractions drive separation along PC2. It is also shown in figure 4.10, that the conjugated fraction is most different from the other two fractions when considering contribution of individual peaks to separation. A more detailed look at the correlation circle, figure 4.11, which shows the effect of each peak on the MFA separation, corroborates individual PCA analyses, in that compounds eluting at 38 and 73.5 minutes in the conjugated fraction as well as ferulic acid in the bound fraction contribute to separation in PC2 which results in distinctions between DZ-01-974 and DZ-Cr-384 from the rest of the tef varieties. An interesting commonality between the three fractions is a peak that elutes around 57 minutes in all three fractions correlating with white varieties.

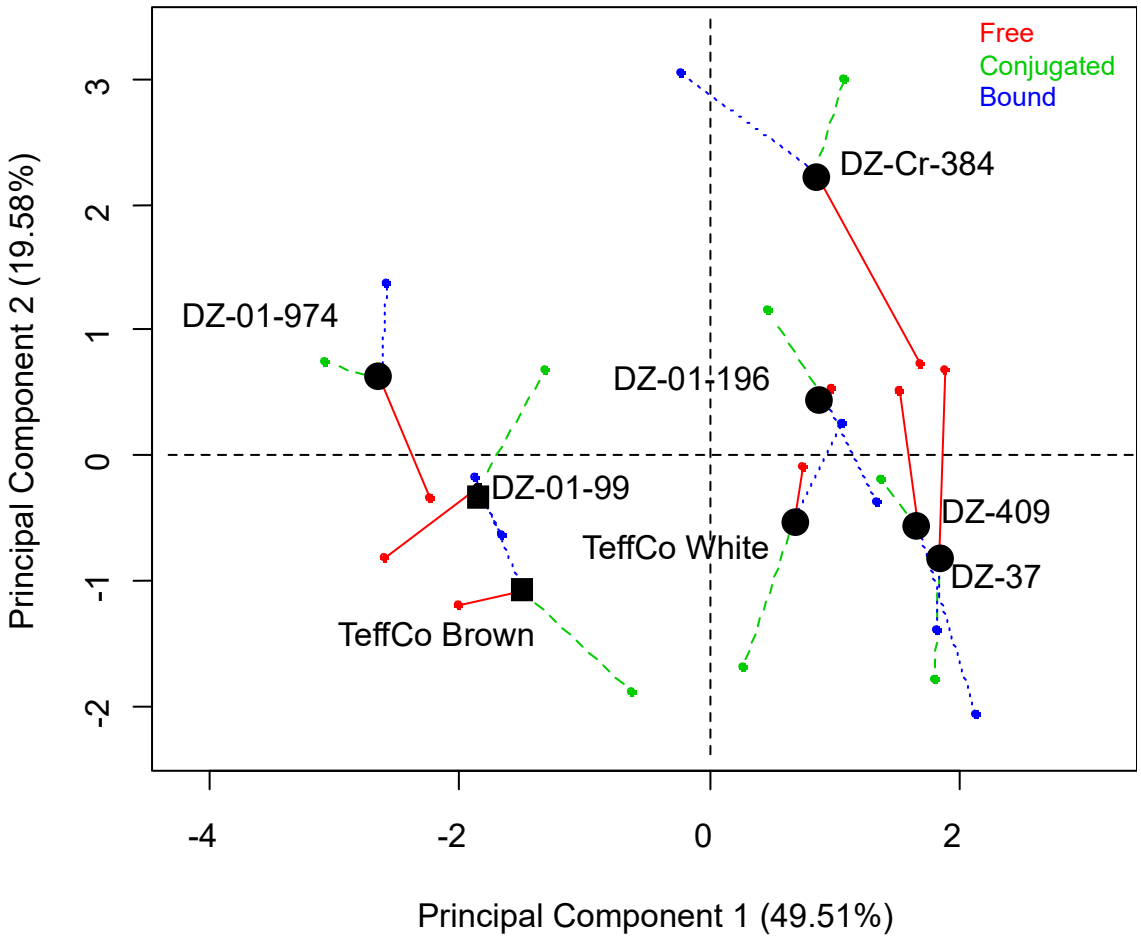


Figure 4.9 Plot of the eight tef varieties as separated by multifactor analysis (MFA) of free (red), conjugated (green), and bound (blue) fraction data (noted by points that branch off from the 'composite' point (black)). Square points indicate brown varieties.

Table 4.3: Contribution of each fraction to variance in the first and second principal component.

	PC1	PC2
Free	36.12	9.71
Conjugated	29.69	49.14
Bound	34.20	41.16

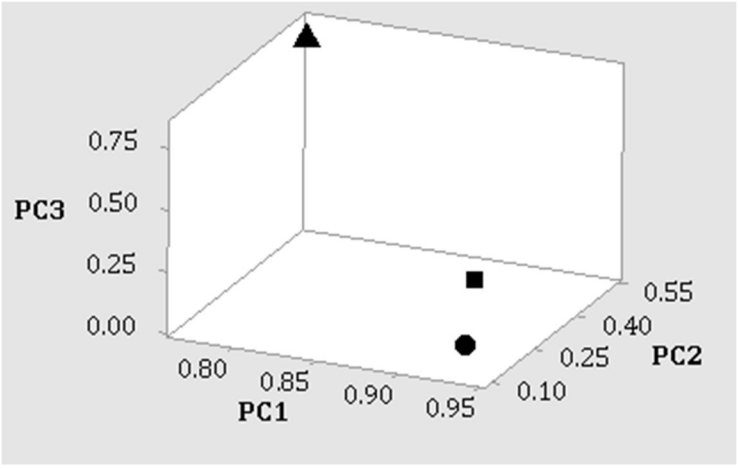


Figure 4.10 3D Plot of relationship between free (circle), conjugated (triangle), and bound (square) groups in each principal component as determined by multifactor analysis (MFA). The bound point is below the free point in the PC 3 dimension.

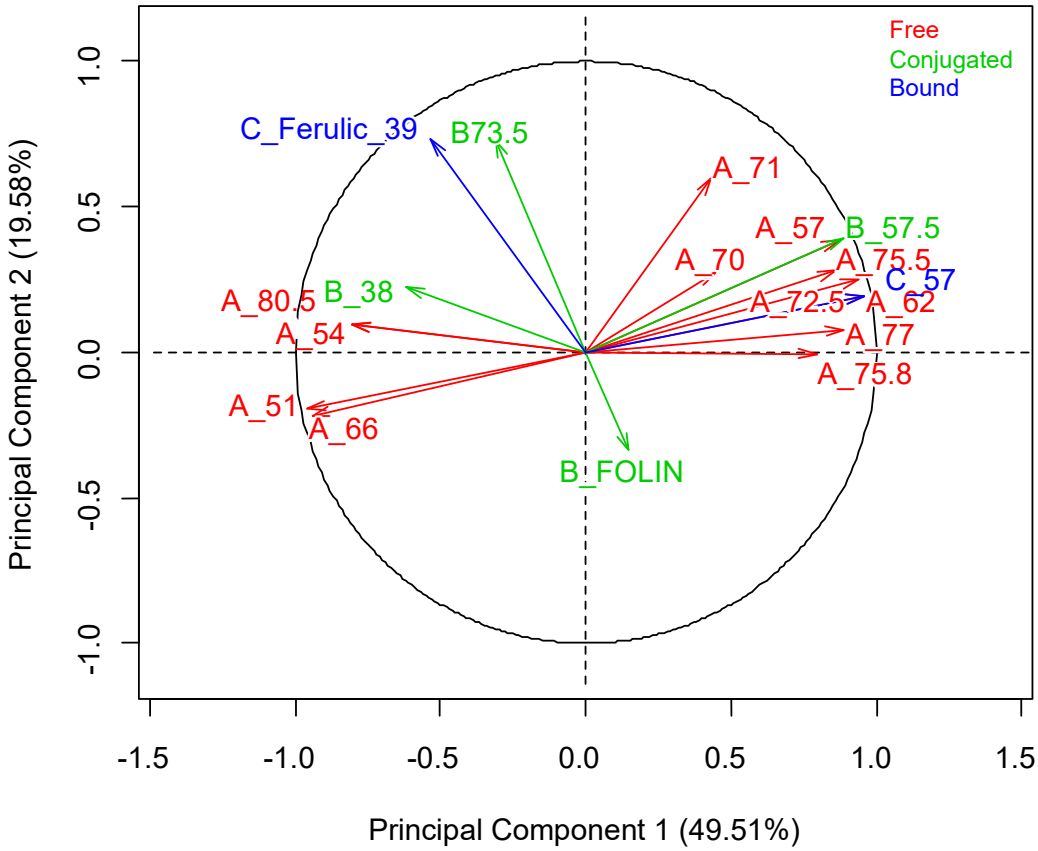


Figure 4.11 Correlation circle, or loading plot, of free (red), conjugated (green), and bound (blue) peaks that correspond to resulting MFA separation in figure 4.9.

4.4 Pigment Assays and Extended Hydrolysis

4.4.1 UV-Vis Detection of 3-deoxyanthocyanidins and Anthocyanidin Extraction

As seen in the UV-Vis spectrum presented in figure 4.12, acidification of methanol extracts of immature tef seeds does not result in a peak at 550 nm when compared to the maize control, which is positive for flavan-4-ols. It may be the case that especially in such a small quantity of seeds as that used in the immature seed extraction, the flavan-4-ols could not be detected. Additionally, in the UV-Vis spectra of immature seed extracts, there is a peak at 425 nm for the brown tef that is not nearly as prominent in the white tef. A 425 nm wavelength corresponds to yellow-orange colors, which are the colors of extracted flavonoid compounds reported in red tef (El-alfy, Ezzat, and Sleem 2012).

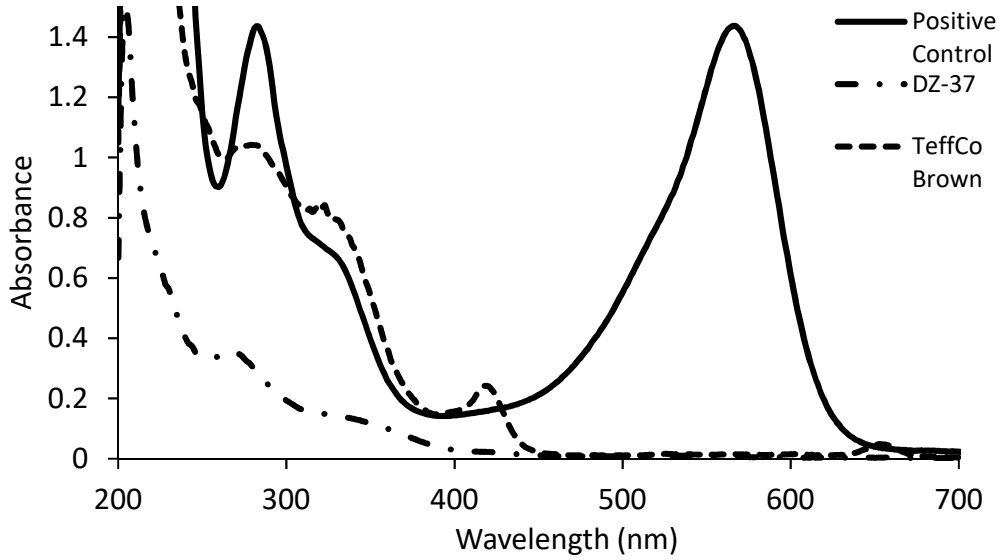


Figure 4.12 Absorbance spectra of acidified (sulfuric acid) methanol extracts of immature TeffCo Brown and DZ-Cr-37 seeds as well as a positive control, purple maize.

It is possible the present compounds responsible for the 425 nm peak in the brown immature seed potentially contribute to pigmentation in brown tef varieties after a chemical

transformation or increase in concentration that occurs as the seed matures. Though UV-Vis analysis of anthocyanidin extracts from mature seeds, shown in 4.13, shows a 550 nm peak present in brown samples that is absent in white samples, the peak is very small. The 3-deoxyanthocyanidins luteolinidin and apigeninidin have absorbance maxima at 490 nm and 476 nm in methanol, and no such peaks are present. Further HPLC analysis of anthocyanidin extracts, found in the appendix figures A.1-A.3, did not detect any anthocyanidin compounds in the samples. From this data it is difficult to conclude that there are anthocyanidin compounds in the brown tef and, if present, they would exist in low concentrations.

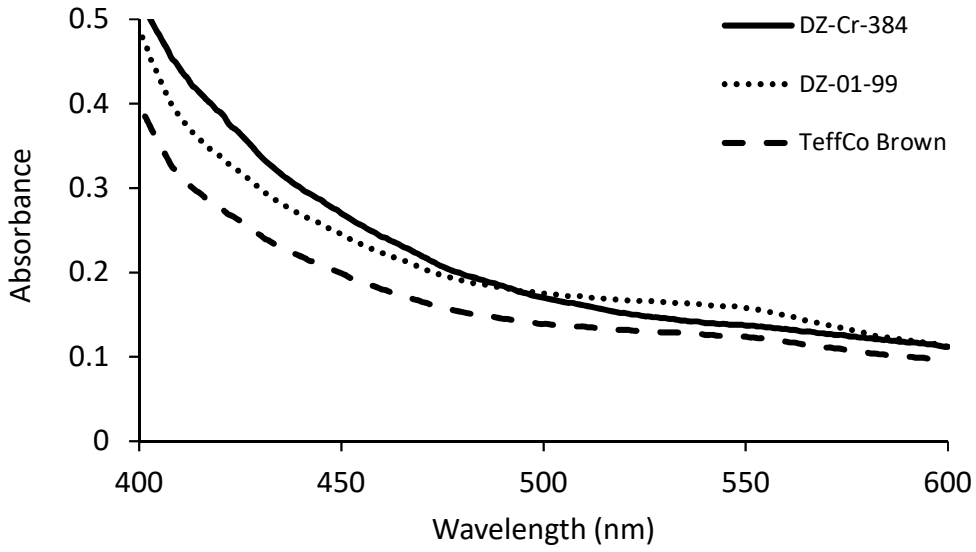


Figure 4.13 Absorbance spectra of tef anthocyanin extracts in methanol (DZ-Cr-384 (white), DZ-01-99 (brown), and TeffCo Brown measured from 400 to 600 nm.

4.4.2 Extended Hydrolysis of Tef Samples

The extended hydrolysis performed on the tef samples to degrade as much of the seed coat material of whole seeds as possible, resulted in nearly full degradation (as determined by

visual observation) of seed coat material after four days (96 h, not shown). Evidence of degradation is exhibited in figures 4.14 and 4.15, where increased hydrolysis resulted in increased yellow pigmentation in the brown and white tef, with more prominent increase in the brown tef.

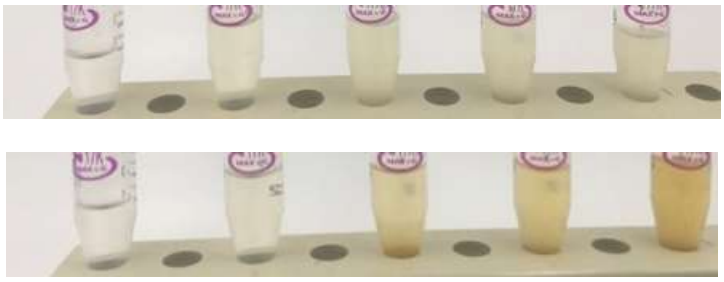


Figure 4.14 Aqueous supernatants of DZ-Cr-37 (top) and DZ-01-99 (bottom) after 0, 1, 10, 24, 40 hours of hydrolysis with 2 M sodium hydroxide (pH ~12.6 in image).

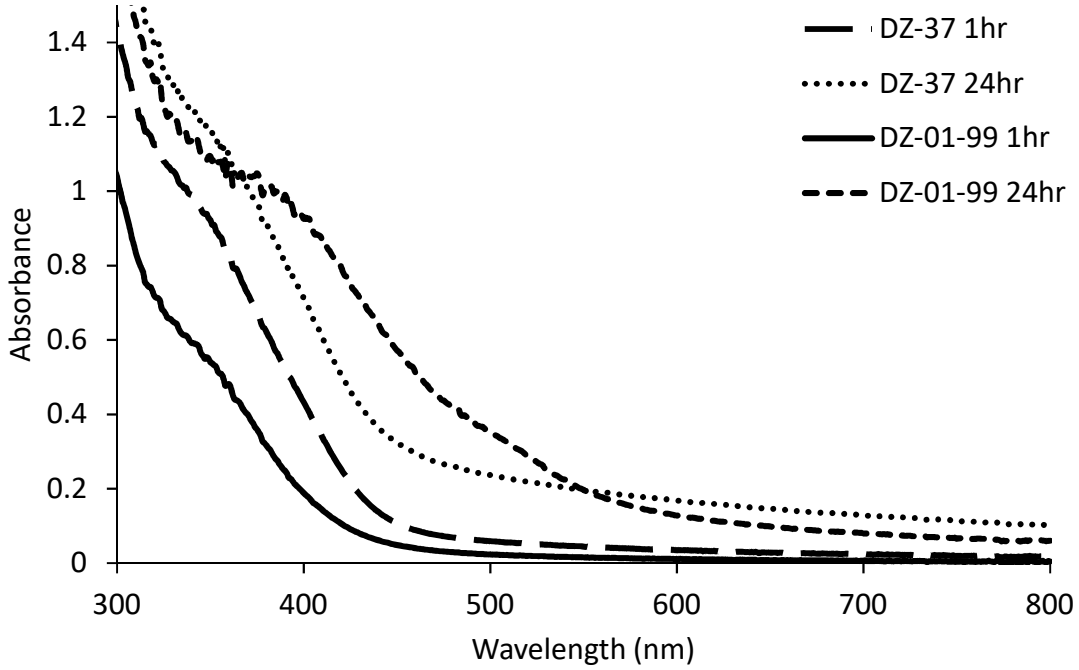


Figure 4.15 Absorbance spectra of aqueous supernatants from DZ-Cr-37 (white) and DZ-01-99 (brown) varieties after 1 and 24 hours of hydrolysis (pH ~ 12.6 at time of analysis).

The supernatant becomes more yellow and UV-Vis peaks at 390 nm and 500 nm increase over time. It should be noted that the yellow color is pH dependent, and therefore likely marks the presence of compounds like anthoxanthins, which are also reported to have the same pH color

dependence in millets (Reichert 1979). It is unclear how these compounds, which are colorless or grey at neutral pH, could contribute to the brown tef color, but it is also possible that other compounds, present in much lower concentrations (as is suggested by anthocyanin analyses and the small 425 nm peak in immature seed extracts) could be more influential on the color differences between varieties. Further analysis of the hydrosylates could offer more insight into the differences in pigmentation between white and brown tef varieties.

4.4.3 Tannin Test

After treatment with bleach (8.25% sodium hypochlorite) and potassium hydroxide solution, tef samples did not present a positive response for tannins. As seen in figure 4.16, before treatment the positive control had a white/grey pericarp that was removed during treatment to reveal a black, pigmented testa layer. None of the tef seeds presented the same response as the positive control. Like the negative control, removal of the pericarp did not reveal a pigmented layer underneath. This was more obvious with the white tef seeds. Brown seeds darkened after treatment, but there was no indication that a pigmented layer had been revealed. Seed coat structure that was not damaged by the bleach treatment appeared the same as the pre-treatment samples. Longer treatment with the bleach resulted in disintegration of the seeds, which is why it could not be left longer to remove the entire seed coat. Upon closer inspection, areas of the brown tef, where the seed coat had been removed, showed yellow/pink color as that of the whites and the negative control. Therefore, these tef varieties do not have a pigmented testa and the pigment in these tef varieties is not likely to be tannin as is found in some sorghums like the positive control.

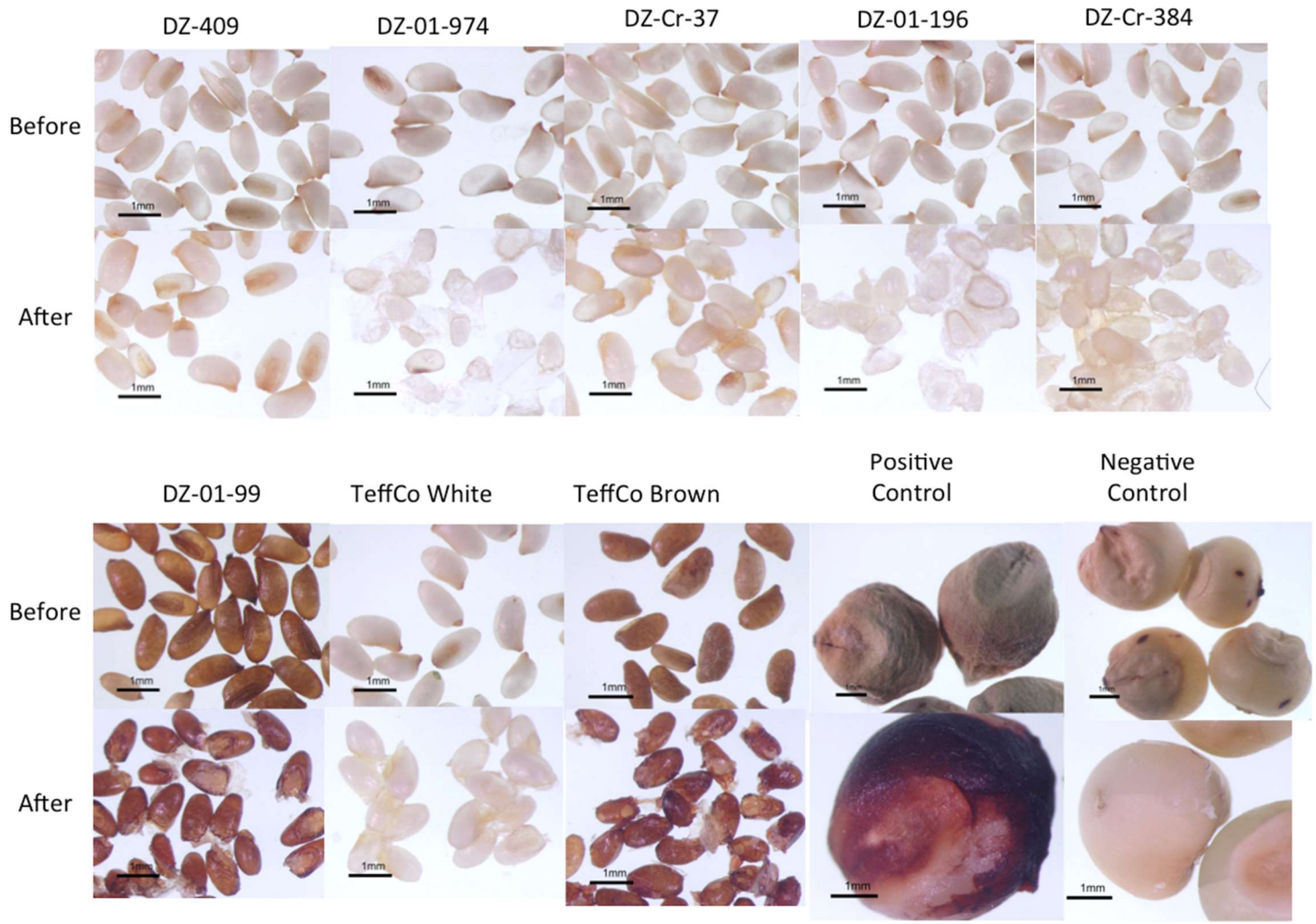


Figure 4.16 Tef varieties and sorghum positive and negative controls before and after bleach/potassium hydroxide treatment.

5. Conclusions and Future Directions

This study focused on analyzing the phenolic profiles of eight tef varieties, six of which were grown under the same conditions, to elucidate differences in phenolic distribution among the free, conjugated, and bound fractions between varieties. Overall, the differences between varieties observed through PCA and MFA analysis show that grouping was able to separate varieties by color (except for DZ-01-974). Additionally, the MFA results suggest that it is important to analyze all three phenolic fractions to understand the similarities and differences between tef varieties. For the present extraction and analysis methodology, phenolic acids do not differ significantly between tef varieties grown in the same location in Ethiopia, nor those grown in the U.S. The only exception is ferulic acid, which has the most effect in the bound fraction and is responsible for setting the DZ-Cr-384 and DZ-01-974 varieties apart from the others in a PCA analysis. The DZ-Cr-384 is a white variety and resulted from the breeding of DZ-01-196 with DZ-01-974. An interesting observation is that DZ-01-196 consistently groups with the white varieties, but DZ-01-974 groups with the browns (most significantly in the free and bound fractions).

Additionally, differences between brown and white varieties are mostly due to compounds eluting toward the end of the reverse phase HPLC chromatograms, suggesting that these are more hydrophobic compounds, such as flavonoids. No connection can be made between 3-deoxyanthocyanins in maize or tannins in sorghum to the compounds responsible for color in brown tef. Further corroboration with pigment analyses suggests that trace amounts flavonoid compounds that absorb light at 425 nm under acidic conditions are responsible for the pigmentation difference.

Future studies on tef and its phenolic profile should aim to further identify compounds, like the peak eluting at 57 minutes in the bound fraction, which play a role in separation of white and brown varieties. Phenolic compounds such as ferulic and vanillic acid are reported to contribute bitter and sour sensations as well as astringency upon consumption (Duizer and Langfried 2016). Understanding the compounds that drive separation between tef varieties, such as the distinction of the popular DZ-Cr-384 variety from others, could support future studies on sensory preferences for white tef and explore if certain compounds influence these preferences. In turn, the differences in phenolic profile are useful information for *in vitro* and *in vivo* studies comparing the physiological effects of test foods or extracts made from brown and white tef varieties which were successfully separated by multivariate analysis. A more complete picture of differences and unique phenolic profiles between varieties, such as higher ferulic acid in DZ-Cr-384 and the compound eluting at 57 minutes that correlates with white varieties, can help with exploring or targeting specific biological effects associated with these compounds while also employing a whole-food matrix.

Additionally, it is still unclear exactly what compounds are responsible for the color difference between white and brown tef varieties and whether this color difference, like in grains such as sorghum and maize, signals the presence of potentially beneficial phenolic compounds like anthocyanidins. Finally, a broader extraction procedure consisting of multiple extraction models for different types of compounds could also offer more insight into the tef phenolic profile, as extraction methods vary greatly and this variation could potentially explain the differences that are observed between studies.

There are differences between the phenolic compounds of white and brown tef, which have been shown in this study as well as others before. However, the differences presented in various studies are not consistent likely due to differences in varieties studied with respect to varieties and growing conditions (both often unspecified) and extraction methods. Even if total

phenolic content does not differ between varieties, the specific phenolic compounds present are different. Therefore, when choosing tef varieties for use in *in vitro* or *in vivo* studies, it is important to pay attention to the fact that even the same varieties may differ in their phenolic content depending on factors such as growing location, storage time, and age. Moreover, specific phenolic profile should be considered alongside total phenolic content, as phenolic content is not enough to capture significant differences between varieties.

References

Ainsworth EA, Gillespie KM. 2007. Estimation of total phenolic content and other oxidation substrates in plant tissues using folin-ciocalteu reagent. Nat Protoc 2(4):875–77. doi:10.1038/nprot.2007.102.

Álvarez P, Alvarado C, Mathieu F, Jiménez L, De La Fuente M. 2006. Diet supplementation for 5 weeks with polyphenol-rich cereals improves several functions and the redox state of mouse leucocytes. Eur J Nutr 45(8):428–38. doi:10.1007/s00394-006-0616-9.

Amoako, DB, Awika JM. 2016. Polymeric tannins significantly alter properties and in vitro digestibility of partially gelatinized intact starch granule. Food Chem 208:10–17. doi:10.1016/j.foodchem.2016.03.096.

An J, Bae I, Han S, Lee S, Lee H. 2016. In vitro potential of phenolic phytochemicals from black rice on starch digestibility and rheological behaviors. J Cereal Sci 70:214–20 doi:10.1016/j.jcs.2016.06.010.

Asenstorfer RE, Wang Y, Mares DJ. 2006. Chemical structure of flavonoid compounds in wheat (Triticum Aestivum L.) flour that contribute to the yellow colour of asian alkaline noodles. J Cereal Sci 43(1):108–19. doi:10.1016/j.jcs.2005.09.001.

Assefa K. 2013. Conventional and Molecular Tef Breeding. In: Assefa K, Chanyalew S, Tadele Z, editors. Achievements and Prospects of Tef Improvement: Proceedings of the Second International Workshop, November 7-9, 2011, Debre Zeit, Ethiopia. Ethiopian Institute of Agricultural Research, Addis Ababa, Ethiopia; Institute of Plant Sciences, University of Bern, Switzerland. Bern, Switzerland: Stämpfli AG. p. 33–51.

Assefa K, Yu JK, Zeid M, Belay G, Tefera H, Sorrells ME. 2010. Breeding tef [*Eragrostis Tef* (Zucc.) Trotter]: conventional and molecular approaches. Plant Breeding 130(1):1–9. doi:10.1111/j.1439-0523.2010.01782.x.

Assefa K, Aliye S, Belay G, Metaferia G, Tefera H, Sorrells ME. 2011. Quncho: the first popular tef variety in Ethiopia. Int J Agric Sustain 9(1):25–34. doi:10.3763/ijas.2010.0545.

Assefa K, Chanyalew S, Tadele Z, editors. 2013. Preface. In: Achievements and Prospects of Tef Improvement: Proceedings of the Second International Workshop, November 7–9, 2011, Debre Zeit, Ethiopia. Ethiopian Institute of Agricultural Research, Addis Ababa, Ethiopia; Institute of Plant Sciences, University of Bern, Switzerland. Bern, Switzerland: Stämpfli AG. p. vii.

Awika JM, Rooney LW, Waniska RD. 2004. Properties of 3-deoxyanthocyanins from sorghum. J Agr Food Chem 52(14):4388–94. doi:10.1021/jf049653f.

Bangoura ML, Nsor-Atindana J, Ming ZH. 2013. Solvent optimization extraction of antioxidants from foxtail millet species' insoluble fibers and their free radical scavenging properties. Food Chem 141(2):736–44. doi:10.1016/j.foodchem.2013.03.029.

Bate-Smith EC, Rasper V. 1969. Tannins of grain sorghum: luteoforol (leucoluteolinidin) 3',4,4',5,7-pentahydroxyflavan. J Food Sci 34:203–9. doi:10.1111/j.1365-2621.1969.tb00919.x.

Baye K. 2014. Teff : nutrient composition and health benefits. Ethiopia Stategy Support Program Working Paper 67. Washington DC: International Food Policy Research Institute. p. 1–18.

Besrat A, Admasu A, Ogbai M. 1980. Critical study of the iron content of tef. Ethiop Med J 18(2):45–52.

Boka B, Woldegiorgis AZ, Haki GD. 2013. Antioxidant properties of ethiopian traditional bread (injera) as affected by processing techniques and tef grain (*Eragrostis tef* (Zucc.)) varieties. Can Chem Trans 1(1):7–24. doi:10.13179/canchemtrans.2013.01.01.0012

Box JD. 1983. Investigation of the folin-ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. Water Res 17(5):511–25. doi:10.1016/0043-1354(83)90111-2.

Bultosa G. 2007. Physicochemical characteristics of grain and flour in 13 tef [Eragrostis Tef (Zucc.) Trotter] grain varieties. JASR 3(12):2042–50.

Bultosa G, Taylor JRN. 2004. Teff. In: Wrigley C, Corke H, Walker CE, editors. *Encyclopedia of Grain Science*. 1st ed. Oxford: Elsevier Ltd. p. 281–90.

Bultosaa G, Hall AN, Taylor JRN. 2002. Physico-chemical characterization of grain tef [Eragrostis Tef (Zucc.) Trotter] starch. *Starch/Stärke* 54:461–68.

Çelik EE, Gökmen V, Fogliano V. 2013. Soluble antioxidant compounds regenerate the antioxidants bound to insoluble parts of foods. J Agr Food Chem 61(43):10329–34. doi:10.1021/jf402523k.

Central Statistical Agency. 2010. Agricultural sample survey: report on area and production of

crops (private peasant holdings, meher season). Statistical bulletin 446. Addis Ababa Ethiopia: Federal Democratic Republic of Ethiopia. p. 1–58.

Chandrasekara A, Naczk M, Shahidi F. 2012. Effect of processing on the antioxidant activity of millet grains. Food Chem 133:1–9. doi:10.1016/j.foodchem.2011.09.043.

Chandrasekara A, Shahidi F. 2010. Content of insoluble bound phenolics in millets and their contribution to antioxidant capacity. J Agr Food Chem 58(11):6706–14. doi:10.1021/jf100868b.

Chandrasekara A, Shahidi F. 2011. Determination of antioxidant activity in free and hydrolyzed fractions of millet grains and characterization of their phenolic profiles by HPLC-DAD-ESI-MS^n. J Funct Food 3(3):144–58. doi:10.1016/j.jff.2011.03.007.

Chanyalew S, Assefa K, Metaferia G. 2013. Phenotypic and molecular diversity in tef. In: Assefa K, Chanyalew S, Tadele Z, editors. Achievements and Prospects of Tef Improvement: Proceedings of the Second International Workshop, November 7-9, 2011, Debre Zeit, Ethiopia. Ethiopian Institute of Agricultural Research, Addis Ababa, Ethiopia; Institute of Plant Sciences, University of Bern, Switzerland. Bern, Switzerland: Stämpfli AG. p. 21–32.

Chen M, McClung AM, Bergman CJ. 2016. Concentrations of oligomers and polymers of proanthocyanidins in red and purple rice bran and their relationships to total phenolics, flavonoids, antioxidant capacity and whole grain color. Food Chem 208:279–87. doi:10.1016/j.foodchem.2016.04.004.

Cheng A, Mayes S, Dalle G, Demissew S, Massawe F. 2015. Diversifying crops for food and nutrition security - a case of teff. Biol Rev 92:188-198. doi:10.1111/brv.12225.

Cherfas, Jeremy. 2015. Millet: how a trendy ancient farm grain turned nomads into farmers. Npr's The Salt. [Accessed 2017 May 24]. http://www.npr.org/sections/thesalt/2015/12/23/460559052/millet-how-a-trendy-ancient-grain-turned-nomads-into-farmers.

Chethan S, Malleshi NG. 2007. Finger millet polyphenols: optimization of extraction and the effect of pH on their stability. Food Chem 105(2):862–70. doi:10.1016/j.foodchem.2007.02.012.

Coghe S, Benoot K, Delvaux F, Vanderhaegen B, Delvaux FR. 2004. Ferulic acid release and 4-vinylguaiacol formation during brewing and fermentation: indications for feruloyl esterase activity in *Saccharomyces Cerevisiae*. J Agr Food Chem 52(3):602–8. doi:10.1021/jf0346556.

Costabile A, Klinder A, Fava F, Napolitano A, Fogliano V, Leonard C, Gibson GR, Tuohy KM. 2008. Whole-grain wheat breakfast cereal has a prebiotic effect on the human gut microbiota: a double-blind, placebo-controlled, crossover study. Brit J Nutr 99(1):110–20. doi:10.1017/S0007114507793923.

Costanza SH, DeWet JMJ, Harlan JR. 1979. Literature review and numerical taxonomy of *Eragrostis tef* (t'ef). Econ Bot 33(4):413–24.

de Oliveira KG, Queiroz AVA, Carlos LA, Cardoso LM, Pinheiro-Sant'Ana HM, Anunciacao PC, Menezes CB de, Silva EC da, and Frederico Barros. 2017. Effect of the storage time and temperature on phenolic compounds of sorghum grain and flour. Food Chem 216:390–98. doi:10.1016/j.foodchem.2016.08.047.

Dolan, JW. 2009. Calibration Curves, Part I: To B or Not to B? *LCGC:* Solutions for Separation Scientists. [Accessed 2017 May 24]. http://www.chromatographyonline.com/calibration-curves-part-i-b-or-not-b.

Dolan, JW. 2010. Column Diameter, Linear Velocity, and Column Efficiency. *LCGC:* Solutions for Separation Scientists. [Accessed 2017 May 28]. http://www.chromatographyonline.com/column-diameter-linear-velocity-and-column-efficiency

Dykes L, Rooney LW. 2007. Phenolic compounds in cereal grains and their health benefits. Cereal Food World 52(3):105-11. doi:10.1094/CFW-52-3-0105.

El-alfy, TS, Ezzat SM, Sleem AA. 2012. Chemical and biological study of the seeds of *Eragrostis tef* (Zucc .) Trotter. Nat Prod Res 26(7):619-29. doi:10.1080/14786419.2010.538924.

Esteban-Torres M, Reverón I, Mancheño JM, De las Rivas B, Muñoz R. 2013. Characterization of a feruloyl esterase from Lactobacillus Plantarum. Appl Environ Microb 79(17):5130–36. doi:10.1128/AEM.01523-13.

Ferede S. 2013. Technological Change and Economic Viability in Tef Prodction. In: Assefa K, Chanyalew S, Tadele Z, editors. Achievements and Prospects of Tef Improvement: Proceedings of the Second International Workshop, November 7-9, 2011, Debre Zeit, Ethiopia. Ethiopian Institute of Agricultural Research, Addis Ababa, Ethiopia; Institute of Plant Sciences, University of Bern, Switzerland. Bern, Switzerland: Stämpfli AG. p. 255–274.

Food and Agriculture Organization of the United Nations. 2017. Country Brief Ethiopia. Global Information and Early Warning System. [Accessed 2017 June 25]. http://www.fao.org/giews/countrybrief/country.jsp?code=ETH .

Forsido SF, Rupasinghe HPV, Astatkie T. 2013. Antioxidant capacity, total phenolics and nutritional content in selected ethiopian staple food ingredients. Int J Food Sci Nutr 64(8):915–20. doi:10.3109/09637486.2013.806448.

Fufa B, Behute B, Simons R, Berhe T. 2011. Tef harvest and processing. In: Tef diagnostic report: strengthening the tef value chain in Ethiopia. Ethiopian Agricultural Transformation Agency. p. 13–16.

Gangopadhyay N, Rai DK, Brunton NP, Gallagher E, Hossain MB. 2016. Antioxidant-guided

isolation and mass spectrometric identification of the major polyphenols in barley (Hordeum Vulgare) grains. Food Chem 210:212–20. doi:10.1016/j.foodchem.2016.04.098.

Gebremariam MM, Zarnkow M, Becker T. 2012. Teff (*Eragrostis tef*) as a raw material for malting, brewing and manufacturing of gluten-free foods and beverages: a review. J Food Sci Tech 51(11):1–15. doi:10.1007/s13197-012-0745-5.

Gómez-Caravaca AM, Verardo V, Berardinelli A, Marconi E, Caboni MF. 2014. A chemometric approach to determine the phenolic compounds in different barley samples by two different stationary phases: a comparison between C18 and pentafluorophenyl core shell columns. J Chromatogr A 1355:134–42. doi:10.1016/j.chroma.2014.06.007.

Grotewold E, Chamberlin M, Snook M, Siame B, Butler L, Swenson J, Maddock S, St. Clair G, Bowen B. 1998. Engineering secondary metabolism in maize cells by ectopic expression of transcription factors. Plant Cell 10(5):721–40. doi:10.1105/tpc.10.5.721.

Gunenc A, HadiNezhad M, Farah I, Hashem A, Hosseinian F. 2015. Impact of supercritical CO_2 and traditional solvent extraction systems on the extractability of alkylresorcinols, phenolic profile and their antioxidant activity in wheat bran. J Funct Food 12:109–19. doi:10.1016/j.jff.2014.10.024.

Guo W, Beta T. 2013. Phenolic acid composition and antioxidant potential of insoluble and soluble dietary fibre extracts derived from select whole-grain cereals. Food Res Int 51(2):518–25. doi:10.1016/j.foodres.2013.01.008.

Harborne JB.1998. Phytochemical Methods. A Guide to Modern Techniques of Plant Analysis. 3rd ed. New York, NY: Champman and Hall. 302 p.

Helbing J. 2009. Confocal laser scanning microscopy and scanning electron microscopy for the observation of the malting of various cereals and pseudo-erals [thesis]. Technical University of Munich. [citation Accessed 2017 May 28]. http://lbgt.wzw.tum.de/index.php?id=86&L=1#c1301

Herald TJ, Gadgil P, Tilley M. 2012. High-throughput micro plate assays for screening flavonoid content and DPPH-scavenging activity in sorghum bran and flour. J Sci Food Agr 92(11):2326–31. doi:10.1002/jsfa.5633.

Hole AS, Rud I, Grimmer S, Sigl S, Narvhus J, Sahlstrøm S. 2012. Improved Bioavailability of Dietary Phenolic Acids in Whole Grain. J Agr Food Chem 60:6369–75. doi.org/10.1021/jf300410h

Hopman E, Dekking L, Blokland M, Wuisman M, Zuijderduin W, Koning F, Schweizer J. 2008. Tef in the diet of celiac patients in the Netherlands. Scand J Gastroentero 43(3):277–82. doi:10.1080/00365520701714871.

International Committee on Harmonization. 1996. Q2B validation of analytical procedures: methodology. Silver Spring MD:United States Food and Drug Administration. 10 p.

Kelemu F, Kebede L. 2013. Some experierience on tef mechanization. In: Assefa K, Chanyalew S, Tadele Z, editors. Achievements and Prospects of Tef Improvement: Proceedings of the Second International Workshop, November 7-9, 2011, Debre Zeit, Ethiopia. Ethiopian Institute of Agricultural Research, Addis Ababa, Ethiopia; Institute of Plant Sciences, University of Bern, Switzerland. Bern, Switzerland: Stämpfli AG. p.161–68.

Kim J, Hyun TK, Kim M. 2011. The inhibitory effects of ethanol extracts from sorghum, foxtail millet and proso millet on α-glucosidase and α-amylase activities. Food Chem 124(4):1647–51. doi:10.1016/j.foodchem.2010.08.020.

Kim KH, Tsao R, Yang R, Cui SW. 2006. Phenolic acid profiles and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. Food Chem 95(3):466–73. doi:10.1016/j.foodchem.2005.01.032.

Kotaskova E, Sumczynski D, Mlcek J, Valasek P. 2016. Determination of free and bound phenolics using HPLC-DAD, antioxidant activity and in vitro digestibility of *Eragrostis tef*. J Food Compos Anal 46:15–21. doi:10.1016/j.jfca.2015.11.001.

Kreitschitz A, Tadele Z, Gola EM. 2009. Slime cells on the surface of Eragrostis seeds maintain a level of moisture around the grain to enhance germination. Seed Sci Res 19(1):27–35.

Lappi J, Aura A, Katina K, Nordlund E, Kolehmainen M, Mykkänen H, Poutanen K. 2013. Comparison of postprandial phenolic acid excretions and glucose responses after Ingestion of breads with bioprocessed or native rye bran. Food Funct 4(6):972–81. doi:10.1039/c3fo60078e.

Li X, Kim J, Park S, Zhao S, Kim Y, Lee S, Park S. 2014. Comparative analysis of flavonoids and polar metabolite profiling of tanno-original and tanno-high rutin buckwheat. J Agr Food Chem 62(12):2701–8. doi:10.1021/jf4049534.

Liyana-Pathirana CM, Shahidi F. 2006. Importance of insoluble-bound phenolics to antioxidant properties of wheat. J Agr Food Chem 54(4):1256–64. doi:10.1021/jf052556h.

Ma D, Sun D, Li Y, Wang C, Xie Y, Guo T. 2014. Effect of nitrogen fertilisation and irrigation on phenolic content, phenolic acid composition, and antioxidant activity of winter wheat grain. J Sci Food Agr 95:1039-46. doi:10.1002/jsfa.6790.

Mamo T, Parsons JW. 1987. Iron nutrition of *Eragrostis tef* (teff). Trop Agr 64 (4): 313–17.

Mateo Anson N, Havenaar R, Bast A, Haenen GRMM. 2010. Antioxidant and anti-inflammatory capacity of bioaccessible compounds from wheat fractions after gastrointestinal digestion. J Cereal Sci 51 (1):110–14. doi:10.1016/j.jcs.2009.10.005.

Mateo Anson N, van den Berg R, Havenaar R, Bast A, Haenenm GRMM. 2009. Bioavailability of ferulic acid is determined by its bioaccessibility. J Cereal Sci 49 (2):296–300. doi:10.1016/j.jcs.2008.12.001.

Miene C, Weise A, Glei M. 2011. Impact of polyphenol metabolites produced by colonic microbiota on expression of COX-2 and GSTT2 in human colon cells (LT97). Nutr Cancer 63(4):653–62. doi:10.1080/01635581.2011.552157.

Monagas M, Khan N, Andrés-Lacueva C, Urpí-Sardá M, Vázquez-Agell M, Lamuela-Raventós RM, Estruch R. 2009. Dihydroxylated phenolic acids derived from microbial metabolism reduce lipopolysaccharide-stimulated cytokine secretion by human peripheral blood mononuclear cells. Brit J Nutr 102:201–6. doi:10.1017/S0007114508162110.

Moore J, Cheng Z, Hao J, Guo G, Liu JG, Lin C, Yu L. 2007. Effects of solid-state yeast treatment on the antioxidant properties and protein and fiber compositions of common hard wheat bran. J Agr Food Chem 55(25):10173–82. doi:10.1021/jf071590o.

Moraes ÉA, Da Silva Marineli R, Lenquiste SA, Steel CJ, De Menezes CB, Queiroz VAV, Júnior MRM. 2015. Sorghum flour fractions: correlations among polysaccharides, phenolic compounds, antioxidant activity and glycemic index. Food Chem 180:116–23. doi:10.1016/j.foodchem.2015.02.023.

Ndolo VU, Beta T, Fulcher RG. 2013. Ferulic acid fluorescence intensity profiles and concentration measured by HPLC in pigmented and non-pigmented cereals. Food Res Int 52(1):108–18. doi:10.1016/j.foodres.2013.02.031.

Nicoletti I, Martini D, De Rossi A, Taddei F, D'Egidio MG, Corradini D. 2013. Identification and quantification of soluble free, soluble conjugated, and insoluble bound phenolic acids in durum wheat (triticum Turgidum L. Var. Durum) and derived products by RP-HPLC on a semimicro separation scale. J Agr Food Chem 61(48):11800–807. doi:10.1021/jf403568c.

Nurse, Earle. 2015. Teff, the Ethiopian superfood that used to be banned. CNN. [Accessed 2017 May 28]. http://www.cnn.com/2015/12/18/africa/ethiopian-superfood-teff/.

Ounnas F, Privé F, Salen P, Gaci N, Tottey W, Calani L, Bresciani L, López-Gutiérrez N, Hazane-Puch F, Laporte F et al. 2016. Whole rye consumption improves blood and liver N-3 fatty acid profile and gut microbiota composition in rats. Plos One 11(2):1–18. doi:10.1371/journal.pone.0148118.

Panato A, Antonini E, Bortolotti F, Ninfali P. 2017. The histology of grain caryopses for nutrient location: a comparative study of six cereals. Int J Food Sci Tech 52(5):1238–45. doi:10.1111/IJFS.13390.

Parker ML, Umeta M, Faulks RM. 1989. The contribution of flour components to the structure of injera, an Ethiopian fermented bread made from tef (*Eragrostis tef*). J Cereal Sci 10(2):93–104. doi:10.1016/S0733-5210(89)80038-4.

Poole, Colin F. 2003. *The Essence of Chromatography*. 1st ed. Amsterdam, NE:Elsevier. 936 p.

Ramos-Escudero F, Muñoz AM, Alvarado-Ortíz C, Alvarado A, Yáñez JA. 2012. Purple corn (Zea Mays L.) phenolic compounds profile and its assessment as an agent against oxidative

stress in isolated mouse organs. J Med Food 15(2):206–15. doi:10.1089/jmf.2010.0342.

Refera A. 2001. TEF: post-harvest operations. Mejia D, Lewis B editors. Addis Ababa, Ethiopia: Institute of Agricultural Research Organization, Holetta Agricultural Research Center. 60 p.

Reichert, R. D. 1979. The pH-sensitive pigments in pearl millet. Cereal Chem 4(56):291–294.

Rezar V, Pajk T, Marinsek Logar R, Jese Janezic V, Salobir K, Oresnik A, Salobir J. 2003. Wheat bran and oat bran effectively reduce oxidative stress induced by high-fat diets in pigs. Ann Nutr Metab 47(2):78–84. doi:10.1159/000069279.

Robbins RJ, Bean SR. 2004. Development of a quantitative high-performance liquid chromatography-photodiode array detection measurement system for phenolic acids. J Chromatogr A 1038:97–105. doi:10.1016/j.chroma.2004.03.009.

Romer N. 2015. "Do you know where your quinoa comes from? *North American Congress on Latin America*. [Accessed 2017 May 28]. http://nacla.org/news/2015/12/23/do-you-know-where-your-quinoa-comes.

Russell WR, Drew JE, Scobbie L, Duthie GG. 2006. Inhibition of cytokine-induced prostanoid biogenesis by phytochemicals in human colonic fibroblasts. BBA-Mol Basis Dis 1762(1):124–30. doi:10.1016/j.bbadis.2005.08.007.

Salawu SO, Bester MJ, Duodu KG. 2014. Phenolic composition and bioactive properties of cell wall preparations and whole grains of selected cereals and legumes. J Food Biochem 38(1):62–72. doi:10.1111/jfbc.12026.

Sanchez D. 2015. Ethiopia lifts ban on teff flour exports with tight controls. [Accessed 2017 May 28]. *AFK Insider*. http://afkinsider.com/95724/ethiopia-lifts-ban-on-teff-flour-exports/.

Saulnier L, Crépeau MJ, Lahaye M, Thibault JF, Garcia-Conesa MT, Kroon PA, Williamson G. 1999. Isolation and structural determination of two 5,5'-diferuloyl oligosaccharides indicate that maize heteroxylans are covalently cross-linked by oxidatively coupled ferulates. Carbohyd Res 320 (1-2):82–92. doi:10.1016/S0008-6215(99)00152-4.

Shao Y, Xu F, Sun X, Bao J, Beta T. 2014. Identification and quantification of phenolic acids and anthocyanins as antioxidants in bran, embryo and endosperm of white, red and black rice kernels (Oryza Sativa L.). J Cereal Sci 59(2):211–18. doi:10.1016/j.jcs.2014.01.004.

Shipman K. 2016. Sorghum an ancient grain poised for new markets. *FarmWeekNow*. [Accessed 2017 May 28]. http://farmweeknow.com/story-sorghum-ancient-grain-poised-new-markets-4-149469.

Shumoy H, Gabaza M, Vandevelde J, Raes K. 2017. Soluble and bound phenolic contents and antioxidant capacity of tef injera as affected by traditional fermentation. J Food Compos Anal 58:52–59. doi:10.1016/j.jfca.2017.01.004.

Shumoy H, Raes K. 2016. Antioxidant potentials and phenolic composition of tef varieties: an

indigenous Ethiopian cereal. Cereal Chem 93(5):465–70. doi:10.1094/CCHEM-10-15-0210-R.

Singleton VL, Rossi JA. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Viticult, 16:144–58. doi:10.12691/ijebb-2-1-5.

Singleton, VL, Orthofer R, Lamuela-Raventos RM. 1998. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol 299:152–78. doi:10.1016/S0076-6879(99)99017-1.

Stalikas, CD. 2007. Extraction, separation, and detection methods for phenolic acids and flavonoids. J Sep Sci 30(18):3268–95. doi:10.1002/jssc.200700261.

Styles DE, Ceska O. 1977. The genetic control of flavanoid synthesis in maize. Can J Genet Cytol 19:289–302.

Subba Rao MVSST, Muralikrishna G. 2002. Evaluation of the antioxidant properties of free and bound phenolic acids from native and malted finger millet (Ragi, *Eleusine Coracana* Indaf-15). J Agr Food Chem 50(4):889–92. doi:10.1021/jf011210d.

Sumczynski D, Kotásková E, Družbíková H, Mlček J. 2016. Determination of contents and antioxidant activity of free and bound phenolics compounds and in vitro digestibility of commercial black and red rice (Oryza Sativa L.) varieties. Food Chem 211:339–46. doi:10.1016/j.foodchem.2016.05.081.

Tang Y, Li X, Zhang B, Chen PX, Liu R, Tsao R. 2015. Characterisation of phenolics, betanins and antioxidant activities in seeds of three chenopodium quinoa willd. genotypes. Food Chem166:380–88. doi:10.1016/j.foodchem.2014.06.018.

Taylor JRN, Duodu KG. 2015. Effects of processing sorghum and millets on their phenolic phytochemicals and the implications of this to the health-enhancing properties of sorghum and millet food and beverage products. J Sci Food Agr 95(2):225–37. doi:10.1002/jsfa.6713.

Tesema A. 2013. Genetic resources of tef in Ethiopia. In: Assefa K, Chanyalew S, Tadele Z, editors. Achievements and Prospects of Tef Improvement: Proceedings of the Second International Workshop, November 7-9, 2011, Debre Zeit, Ethiopia. Ethiopian Institute of Agricultural Research, Addis Ababa, Ethiopia; Institute of Plant Sciences, University of Bern, Switzerland. Bern, Switzerland: Stämpfli AG. p. 15–20.

Tuohy KM, Conterno L, Gasperotti M, Viola R. 2012. Up-regulating the human intestinal microbiome using whole plant foods, polyphenols, and/or fiber. J Agr Food Chem 60(36):8776–82. doi:10.1021/jf2053959.

Umeta M, Parker ML. 1996. Microscopic studies of the major macro-components of seeds, dough, and injera from tef (*Eragrostis Tef*). Sinet: Ethiop J Sci 19(1):141–48.

Umeta M, Faulks RM. 1988. The effect of fermentation on the carbohydrates in tef (*Eragrostis tef*). Food Chem 27(3):181–89. doi:10.1016/0308-8146(88)90061-1.

Upadhyay R, Jha A, Singh SP, Kumar A, Singh M. 2015. Appropriate solvents for extracting total phenolics, flavonoids and ascorbic acid from different kinds of millets. J Food Sci Tech 52(1):472–78. doi:10.1007/s13197-013-0976-0.

USDA. 2015. Dietary Guidelines for Americans 2015-2020. [Accessed 2017 May 28] http://health.gov/dietaryguidelines/2015/guidelines/acknowledgments/.

Vavilov NI. 1951. The origin, variation, immunity and breeding of cultivated plants. Starrchester K, translator. New York: Roland Press. 364.

Van Rymenant E, Abranko L, Tumova S, Grootaert C, Van Camp J, Williamson G, Kerimi A. 2017. Chronic exposure to short-chain fatty acids modulates transport and metabolism of microbiome-derived phenolics in human intestinal cells. J Nutr Biochem 39:156–68. doi:10.1016/j.jnutbio.2016.09.009.

Verma B, Hucl P, Chibbar RN. 2008. Phenolic content and antioxidant properties of bran in 51 wheat cultivars. Cereal Chem 85(4):544–49. doi:10.1094/CCHEM-85-4-0544.

Vitaglione P, Napolitano A, Fogliano V. 2008. Cereal dietary fibre: a natural functional ingredient to deliver phenolic compounds into the gut. Trends Food Sci Tech 19(9):451–63. doi:10.1016/j.tifs.2008.02.005.

Waniska RD, Hugo LF, Rooney LW. 1992. Practical methods to determine the presence of tannins in sorghum. J Appl Poultry Res 1:122–28.

Waterhouse AL. 2001. Determination of total phenolics. In: Wrolstad RE, editor. Current protocols in food analytical chemistry. New York:Wiley. p. I1.1–I1.1.8.

Wehrens R. 2011. Chemometrics with R: Multivariate Data Analysis in the Natural Sciences and Life Sciences. In: Use R!, Gentleman R, Hornik K, Parmigiani G, editors. Berlin Heidelberg: Springer-Verlag. p. 43–66.

Whole Grains Council. 2013. Definition of whole grains. [Accessed 2017 May 28]. http://wholegrainscouncil.org/whole-grains-101/definition-of-whole-grain.

Yang F, Li W, Jiang N, Yu H, Morohashi K, Ouma WZ, Morales-Mantilla DE, Gomez-Cano FA, Mukundi E, Prada-Salcedo LD, et al. 2016. A maize gene regulatory network for phenolic metabolism. Mol Plant 10(3):498–515. doi:10.1016/j.molp.2016.10.020.

Yang Z, Zhai W. 2010. Identification and antioxidant activity of anthocyanins extracted from the seed and cob of purple corn (*Zea Mays* L.). Innov Food Sci Emerg 11(1):169–76. doi:10.1016/j.ifset.2009.08.012.

Zhang A, Wan L, Wu C, Fang Y, Han G, Li H, Zhang Z, Wang H. 2013. Simultaneous determination of 14 phenolic compounds in grape canes by HPLC-DAD-UV using

wavelength switching detection. Molecules 18(11):14241–57. doi:10.3390/molecules181114241.

Zhang, M, Zhang R, Zhang F, Liu R. 2010. Phenolic profiles and antioxidant activity of black rice bran of different commercially available varieties. J Agr Food Chem 58(13):7580–87. doi:10.1021/jf1007665.

Appendix

This anthocyanin HPLC method as developed by Dr. Iffa Gaffoor and Dr. Surinder Chopra from the Pennsylvania State University Plant Science Department: Samples are analyzed with a Shimadzu LC-20AT Prominence liquid chromatograph with SPD-M20 DAD and Asentis RP-amide C18 column (4.6 mm x 250 mm, Supelco, Bellefonte, PA). The method consists of a linear gradient from 5% -80% methanol (0.2% formic acid) for 20 minutes, with a 5 minutes flush with 100% methanol and 9 minute conditioning; all at 0.8 ml/min. Absorbance was monitored from 200 nm to 800 nm.

Table A.1: Aligned (by approximate (app.) retention time (RT)) HPLC peak areas for free (A), conjugated (B), and bound fractions (C) respectively (DZ-409 = 1; DZ-01-974 = 2; DZ-Cr-37 = 3; DZ-01-196 = 4; DZ-Cr-384 = 5; DZ-01-99 = 6; TeffCo White = 7; TeffCo Brown = 8). Peaks are given as peak areas. Zero peaks are entered as 0.01.

Peak	App. RT	A1 Rep1	A1 Rep3	A2 Rep1	A2 Rep2	A3 Rep1	A3 Rep2	A4 Rep1	A4 Rep2	A4 Rep3	A5 Rep1
A1	5.5	13.19	12.43	12.42	7.20	16.41	7.95	11.38	7.24	5.47	9.04
A_Protocat	9	25.60	27.03	19.39	36.06	43.87	9.78	38.97	66.26	28.66	68.51
A3	10	10.73	11.91	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
A4	11	55.89	39.98	47.54	25.86	68.39	34.42	32.20	22.43	26.37	43.63
A5	12	51.05	65.01	32.14	24.26	58.05	35.73	31.24	19.26	20.84	55.76
A_Benzoic	14	85.38	65.66	66.92	54.79	55.67	52.80	92.42	65.61	81.06	62.62
A7	16	56.10	58.09	39.50	37.50	43.86	30.45	56.09	27.07	46.79	32.51
A_Vanillic	18	96.17	118.27	88.54	73.39	130.74	81.19	125.26	74.34	100.32	101.08
A9	20	20.77	33.87	9.62	15.21	9.73	11.22	17.08	19.24	19.11	0.01
A_Syringic	22	49.50	64.54	32.49	23.94	76.61	34.82	78.40	39.07	44.99	46.57
A11	28	12.00	14.06	21.31	25.63	0.01	0.01	0.01	0.01	0.01	0.01
A12	30	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
A_pCoum.	33	23.04	29.85	24.78	21.70	21.24	14.79	29.21	16.75	24.55	21.86
A14	33.5	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
A_Ferulic	37	227.39	233.08	360.01	255.19	274.24	149.79	350.71	175.53	254.94	263.90
A16	39	90.44	57.85	101.65	77.95	91.03	28.84	115.68	64.85	70.17	73.11
A17	51	0.01	0.01	1541.87	1263.58	0.01	0.01	484.96	273.66	432.02	121.23
A18	51	111.05	136.44	0.01	0.01	136.42	106.45	0.01	0.01	0.01	0.01
A19	54	0.01	0.01	198.68	134.79	0.01	0.01	170.41	41.79	108.93	0.01
A20	54	89.59	71.41	0.01	0.01	94.10	36.31	0.01	0.01	0.01	0.01

Table A.1 Continued

Peak	App. RT	A1 Rep1	A1 Rep3	A2 Rep1	A2 Rep2	A3 Rep1	A3 Rep2	A4 Rep1	A4 Rep2	A4 Rep3	A5 Rep1
A21	57	3304.24	3498.91	593.55	462.70	3969.60	2715.40	3495.75	1835.66	2813.00	3756.33
A22	62	259.08	346.42	0.01	0.01	347.99	185.95	401.57	143.75	215.57	235.95
A23	66	0.01	0.01	241.60	196.24	0.01	0.01	0.01	0.01	0.01	0.01
A24	70	233.56	225.99	226.76	204.26	515.10	360.33	347.27	161.83	265.88	237.86
A25	71	263.89	254.84	191.87	155.81	410.04	309.77	361.26	166.41	274.77	393.86
A26	72.5	478.30	508.47	28.12	19.98	576.27	397.59	586.75	275.23	472.30	506.34
A27	74	172.56	146.72	0.01	0.01	232.44	125.02	185.15	90.23	136.96	159.61
A28	74.5	0.01	0.01	0.01	0.01	9.33	9.08	0.01	32.70	53.22	0.01
A29	75.5	144.36	237.22	72.46	46.62	229.61	172.19	59.94	171.16	159.10	155.85
A30	75.8	290.47	335.24	68.51	52.33	571.10	371.53	313.50	0.01	90.32	168.29
A31	76.4	16.23	42.94	62.75	43.45	17.13	9.80	23.95	18.23	62.25	12.63
A32	76.8	0.01	0.01	0.01	0.01	22.00	12.08	0.01	0.01	0.01	0.01
A33	77	795.70	807.59	199.87	157.77	987.40	676.35	739.29	371.73	79.49	716.26
A34	78	638.79	698.30	610.24	491.69	635.31	427.49	1081.62	437.74	638.09	822.33
A35	78.8	931.22	1011.18	885.64	662.17	1010.70	621.79	267.64	604.92	851.63	1091.32
A36	79	259.53	220.75	166.58	82.54	316.72	158.12	18.38	126.29	174.28	235.63
A37	80.2	14.01	10.58	133.51	103.61	17.75	7.50	7.19	7.41	16.55	13.85
A38	80.5	63.63	52.71	244.65	193.55	8.97	2.57	0.01	0.01	0.01	72.81
A39	81	30.62	19.58	147.59	115.35	51.73	38.12	38.61	26.90	29.34	55.77
A40	81.8	21.64	16.83	0.01	0.01	43.95	19.71	23.31	14.46	22.11	0.01
Peak	App. RT	A5 Rep2	A6 Rep1	A6 Rep2	A6 Rep3	A7 Rep1	A7 Rep2	A7 Rep3	A 8 Rep1	A 8 Rep2	A 8 Rep3
A1	5.5	6.91	13.33	5.70	7.50	0.01	0.01	0.01	0.01	0.01	0.01
A_Protocat	9	36.67	116.28	254.97	112.28	34.43	29.86	21.63	43.06	33.49	62.24
A3	10	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
A4	11	27.05	145.69	122.07	113.06	31.74	29.36	40.37	52.28	39.78	77.76

Table A.1 Continued

Peak	App. RT	A5 Rep2	A6 Rep1	A6 Rep2	A6 Rep3	A7 Rep1	A7 Rep2	A7 Rep3	A 8 Rep1	A 8 Rep2	A 8 Rep3
A5	12	29.50	27.02	19.31	21.55	27.22	25.60	27.81	17.16	0.01	25.37
A_Benzoic	14	49.93	43.37	28.86	34.07	48.39	25.43	56.56	11.33	0.01	20.68
A7	16	21.05	26.14	21.77	26.70	29.84	33.29	41.67	19.09	15.66	19.87
A_Vanillic	18	69.87	73.22	61.13	53.14	66.84	61.69	84.21	28.92	25.93	33.67
A9	20	0.01	0.01	55.96	15.09	0.01	0.01	0.01	0.01	0.01	0.01
A_Syringic	22	31.29	27.08	19.95	16.12	57.50	50.89	70.92	22.46	18.59	26.52
A11	28	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
A12	30	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
A_pCoum.	33	16.48	18.75	15.56	17.00	0.01	0.01	0.01	0.01	0.01	0.01
A14	33.5	0.01	0.01	0.01	0.01	27.25	22.78	35.32	0.01	0.01	0.01
A_Ferulic	37	164.99	240.41	206.87	212.56	198.79	157.57	248.12	156.46	99.29	207.03
A16	39	32.02	38.66	60.04	49.34	95.12	66.06	97.89	45.33	32.08	53.40
A17	51	85.73	1978.53	1574.57	1403.33	133.62	152.28	146.13	1601.32	1318.22	2267.90
A18	51	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
A19	54	0.01	381.88	199.99	112.44	0.01	0.01	0.01	90.74	74.12	80.32
A20	54	0.01	0.01	0.01	0.01	17.23	15.00	0.01	0.01	0.01	0.01
A21	57	2686.07	424.52	331.76	252.40	1534.96	1513.91	2112.71	282.42	215.47	377.12
A22	62	141.66	106.65	53.73	82.71	212.76	122.59	175.67	0.01	0.01	0.01
A23	66	0.01	218.67	169.34	159.56	0.01	0.01	0.01	191.23	144.14	269.81
A24	70	150.64	165.97	153.85	108.59	155.76	133.10	191.24	36.18	0.01	54.77
A25	71	271.48	0.01	0.01	0.01	109.67	102.47	143.02	40.89	60.88	46.97
A26	72.5	349.36	17.36	21.96	20.54	330.78	316.55	446.25	0.01	0.01	0.01
A27	74	96.47	0.01	0.01	0.01	94.57	77.05	116.65	0.01	0.01	0.01
A28	74.5	0.01	0.01	0.01	0.01	15.43	15.04	22.87	0.01	0.01	0.01
A29	75.5	147.42	96.90	100.50	65.04	139.15	162.07	240.40	43.93	32.31	48.47
A30	75.8	141.41	24.55	0.01	35.23	239.04	184.19	269.50	61.20	42.44	89.09

Table A.1 Continued

Peak	App. RT	A5 Rep2	A6 Rep1	A6 Rep2	A6 Rep3	A7 Rep1	A7 Rep2	A7 Rep3	A 8 Rep1	A 8 Rep2	A 8 Rep3
A31	76.4	6.93	57.31	40.03	43.50	17.28	13.90	19.85	12.77	5.99	16.23
A32	76.8	0.01	0.01	0.01	0.01	22.92	11.46	19.22	0.01	0.01	0.01
A33	77	489.89	417.20	305.73	286.76	424.83	359.71	525.84	278.23	220.68	369.11
A34	78	580.33	515.44	444.70	381.91	379.93	433.84	584.23	387.91	329.28	547.37
A35	78.8	729.75	623.04	518.56	457.90	560.27	591.53	845.11	534.51	422.50	730.28
A36	79	126.60	114.47	115.68	76.58	134.75	109.11	181.89	71.50	44.42	85.88
A37	80.2	9.80	112.39	85.23	78.29	0.01	0.01	0.01	36.83	26.05	49.64
A38	80.5	41.51	178.52	144.50	142.99	0.01	0.01	0.01	44.78	34.23	61.51
A39	81	23.19	68.87	55.66	67.78	38.00	30.42	56.92	25.39	23.40	43.55
A40	81.8	0.01	21.28	0.01	8.40	15.91	11.02	18.35	0.01	0.01	0.01

Peak	App. RT	B1 Rep2	B1 Rep3	B2 Rep1	B2 Rep2	B2 Rep3	B3 Rep1	B3 Rep2	B3 Rep3	B4 Rep1	B4 Rep2	B4 Rep3
B2	6.5	9.83	4.47	22.72	19.67	17.34	17.84	16.90	0.01	7.73	19.83	18.14
BProtocat.	8	57.02	65.29	44.53	43.86	63.86	63.99	54.21	56.45	27.49	56.34	44.11
B4	9	25.55	37.09	29.51	40.99	109.74	36.83	23.48	46.78	33.93	66.43	38.46
B5	11.5	101.94	144.04	103.02	39.46	129.58	121.30	99.23	102.21	122.94	150.88	129.59
B6	12	168.67	203.68	378.01	178.01	609.16	152.73	107.44	192.20	234.14	129.32	290.05
BBenzoic	14	409.55	382.34	172.38	485.50	438.90	315.28	450.57	334.44	344.97	308.54	349.91
B8	15	49.80	102.50	145.72	71.09	144.64	83.02	75.53	55.20	102.47	155.52	109.77
B9	16	104.14	154.80	161.60	106.57	248.65	153.29	99.76	94.61	166.53	202.05	133.09
BVanillic	18	850.56	1012.86	836.27	747.85	1121.22	948.87	980.27	961.84	853.97	685.49	944.10
B12	20.5	70.15	51.63	56.52	82.39	75.39	36.21	37.10	33.57	34.33	66.72	60.45
BSyringic	22.5	573.72	724.23	860.96	619.95	1138.30	626.42	659.56	657.22	791.65	573.93	754.01
B13	24	16.61	26.99	0.01	0.01	0.01	11.58	12.69	19.62	0.01	0.01	0.01
B14	30	67.19	80.39	67.11	75.02	94.92	64.18	64.83	63.12	104.15	52.63	72.61
BpCoum.	33	140.64	210.55	181.29	129.89	207.98	156.49	142.03	150.95	232.18	159.05	173.69
B16	36.5	32.52	33.09	34.26	28.94	18.47	0.01	0.01	0.01	0.01	0.01	0.01

Table A.1 Continued

Peak	App. RT	B1 Rep2	B1 Rep3	B2 Rep1	B2 Rep2	B2 Rep3	B3 Rep1	B3 Rep2	B3 Rep3	B4 Rep1	B4 Rep2	B4 Rep3
B17	38	93.69	76.21	117.72	144.51	178.15	55.16	86.87	76.45	67.84	52.63	110.13
BFerulic	39	156.52	263.77	413.76	209.83	449.13	219.88	168.14	193.58	339.58	250.90	225.69
B19	50	0.01	0.01	0.01	0.01	0.01	80.49	86.22	0.00	72.73	63.24	0.01
B20	57.5	731.55	565.29	92.13	107.61	128.23	618.69	642.67	514.52	610.54	251.81	477.53
B21	70.5	78.50	77.01	30.25	34.05	33.16	125.35	133.51	77.43	98.64	81.93	60.36
B22	71	80.71	57.63	49.26	98.39	113.40	118.78	141.88	70.72	73.55	101.48	45.55
B23	72	10.38	29.71	26.36	25.78	50.19	30.22	33.61	28.98	35.41	27.62	30.48
B24	72.5	0.01	0.01	9.40	16.46	18.50	0.01	0.01	0.01	0.01	0.01	0.01
B25	73.5	114.24	153.63	207.98	163.92	237.08	114.82	111.51	111.89	160.57	117.88	145.32
B26	75	32.44	32.64	41.90	34.18	49.57	27.57	30.36	28.59	38.00	30.99	40.40
B27	76	194.96	97.88	23.08	26.81	39.79	247.21	310.03	119.48	44.41	56.49	30.84
B28	76.8	120.89	158.65	172.14	131.88	229.72	230.84	227.05	193.39	184.27	142.04	159.96
B29	78	6.36	10.29	11.25	18.69	24.75	11.16	21.99	26.98	10.68	7.99	23.83

Peak	App. RT	B5 Rep1	B5 Rep2	B5 Rep3	B6 Rep1	B6 Rep2	B6 Rep3	B8 Rep1	B8 Rep2	B8 Rep3	B9 Rep1	B9 Rep2	B9 Rep3
B2	6.5	26.82	0.01	22.23	14.79	0.01	0.01	11.99	3.84	9.97	0.01	18.01	21.07
BProtocat.	8	93.83	52.87	54.11	59.09	76.89	65.67	41.67	26.01	19.59	104.25	57.25	72.57
B4	9	96.75	46.23	46.56	21.48	149.45	35.37	40.01	47.66	38.98	29.80	20.84	34.74
B5	11.5	160.49	90.66	120.28	66.39	91.77	79.10	100.21	134.80	161.26	41.17	28.65	26.02
B6	12	381.00	107.62	322.42	71.90	175.72	357.93	147.14	179.26	306.79	86.70	207.81	410.26
BBenzoic	14	399.19	361.40	398.96	270.03	358.07	348.43	136.40	349.14	423.31	127.31	300.84	337.80
B8	15	187.69	80.23	92.56	102.49	54.74	92.60	101.31	109.49	124.39	87.00	33.80	60.94
B9	16	249.83	98.47	164.79	80.66	111.74	93.71	90.52	173.86	219.93	61.75	39.37	52.70
BVanillic	18	1017.52	834.37	1046.74	1029.89	1092.30	1200.36	660.52	696.59	707.47	1043.03	954.95	1187.58
B12	20.5	48.20	33.32	49.22	0.00	61.54	29.06	96.60	80.02	79.09	0.01	6.13	5.92
BSyringic	22.5	799.49	637.81	806.59	676.12	625.68	664.58	436.42	475.85	525.44	852.37	510.52	804.45
B13	24	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

Table A.1 Continued

Peak	App. RT	B5 Rep1	B5 Rep2	B5 Rep3	B6 Rep1	B6 Rep2	B6 Rep3	B8 Rep1	B8 Rep2	B8 Rep3	B9 Rep1	B9 Rep2	B9 Rep3
B14	30	64.24	57.25	74.67	46.28	46.45	55.49	59.42	53.60	58.40	25.10	28.38	28.87
BpCoum.	33	239.42	173.57	174.15	130.26	117.99	120.43	125.28	136.27	131.39	76.06	48.20	67.53
B16	36.5	63.55	24.89	45.18	16.49	20.28	16.76	0.01	0.01	0.01	12.46	9.07	11.72
B17	38	70.90	81.08	93.65	37.74	72.91	88.08	114.79	96.04	92.59	29.46	39.76	53.80
BFerulic	39	374.81	253.34	298.50	246.25	186.40	214.52	142.21	241.02	238.71	175.67	85.39	152.82
B19	50	168.26	0.01	60.68	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
B20	57.5	1271.98	813.35	884.27	91.07	114.59	70.87	329.82	357.42	478.66	68.71	41.85	62.81
B21	70.5	140.39	79.41	98.01	18.16	49.61	20.89	28.33	44.07	69.22	9.58	10.49	10.91
B22	71	181.97	117.51	125.71	10.64	18.48	9.20	20.89	27.42	39.74	17.65	13.42	14.04
B23	72	56.10	43.69	43.09	21.57	24.49	18.65	15.35	20.07	19.98	8.55	8.86	10.14
B24	72.5	0.01	0.01	0.01	0.00	2.55	2.93	0.01	0.01	0.01	9.35	8.85	8.93
B25	73.5	171.20	130.16	153.77	138.71	182.11	183.32	77.67	84.79	86.61	68.62	68.06	90.02
B26	75	42.09	29.70	35.54	29.65	21.84	21.09	15.91	13.35	13.53	12.15	11.25	5.27
B27	76	156.44	74.88	94.06	10.71	7.08	13.05	73.52	74.77	164.47	12.13	4.10	13.57
B28	76.8	163.45	123.85	150.28	177.36	157.36	171.91	139.85	144.07	187.12	202.15	123.08	195.68
B29	78	7.24	25.55	16.62	15.52	43.09	31.63	31.02	26.24	17.17	30.83	53.12	38.63

Peak	App. RT	C1 Rep1	C1 Rep2	C1 Rep3	C2 Rep1	C2 Rep2	C2 Rep3	C3 Rep1	C3 Rep2	C3 Rep3	C4 Rep1	C4 Rep2
C1	7	97.95	73.24	71.14	41.92	155.9	4.95	0.01	0.01	0.01	53.62	40.86
C_Benzoic	14	65.83	227.26	221.48	67.97	225.98	208.35	182.68	214.45	211.49	197.27	231.63
C3	16	36.34	11.9	30.2	17.86	45.03	38.54	17.15	13.73	35.84	28.25	24.49
C_Vanillic	18	62.93	48.01	55.36	54.64	50.25	55.68	72.95	49.43	51.8	41.75	43.93
C5	21	10.24	3.3	26.29	21.89	18.24	23.16	10.3	0.01	33.59	21.27	4.23
C_Syringic	22	22.71	31.16	29.62	59.43	49.88	52.82	30.29	34.58	33.92	34.78	36.25
C7	30	63.69	30.29	80.51	65.69	72.67	79.4	59.98	75.34	86.26	71.42	90.98
C_pCoum.	33	227.78	225.38	272.82	273.1	269.45	258.77	204.32	227.89	200.64	227.2	265.97
C_9	38	479.44	471	711.93	712.61	642.99	662.73	413.97	563.27	643.35	309.06	660.88

Table A.1 Continued

Peak	App. RT	C1 Rep1	C1 Rep2	C1 Rep3	C2 Rep1	C2 Rep2	C2 Rep3	C3 Rep1	C3 Rep2	C3 Rep3	C4 Rep1	C4 Rep2
C_Ferulic	39	1821.61	1726.7	2047.63	2497.63	2180.22	2144.99	1855.41	1901.65	1713.56	1876.47	2034.68
C_11	57	194.48	103.42	216.67	0.01	76.82	83.25	181.7	179.23	189.5	173.62	211.14
C_11.5	57_	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
C_12	73.5	23.04	17.35	30.6	50.98	37.84	41.24	20.66	23.47	25.29	24.33	30.52
C_13	74.5	21.67	19.52	18.81	19.5	31.32	16.66	24.73	29	26.64	0.01	0.01
C_13.5	75.5	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
C_14	77	9.65	0.01	10.06	6.23	8.87	4.18	15.51	14.28	18.04	12	9.05
C_16	78	191.57	148.12	218.82	332.42	250.93	265.69	196.42	193.57	181.03	209.34	209
C_17	78.5	17.89	24.57	30.4	23.34	43.3	38.96	15.34	32.98	32.71	8.26	34.22
C_18	78.8	7.18	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
C_19	79	52.74	52.05	68.21	89.51	77.96	72.93	56.74	59.16	56.05	61.81	61.2
C_20	80	10.74	6.81	12.04	16.04	8.24	11.79	11.75	11.09	4.91	5.84	6.34
C_21	80.5	29.86	30.86	40.99	42.66	53.03	45.74	30.69	41.77	33.12	32.69	44.24
C_22	81.4	11.26	5.91	11.43	19.56	16.26	15.4	10.12	10.45	8.39	13.65	11.25
C_23	82	14.81	30.45	22.51	10.48	23.7	17.49	14.49	21.42	22.49	16.09	25.27
C_24	82.5	7.14	0.01	6.83	8.91	6.65	9.69	6.75	5.72	7.31	8.14	5.35
C_25	83	0.01	0.01	0.01	3.95	7.29	7.15	4.22	4.84	3.97	0.01	0.01
C_26	83.5	11.48	58.74	13.16	9.52	6.96	6.05	14.02	6.91	5.6	10.63	5.63

Peak	App. RT	C5 Rep1	C5 Rep2	C5 Rep3	C6 Rep1	C6 Rep2	C6 Rep3	C7 Rep1	C7 Rep2	C7 Rep3	C8 Rep1	C8 Rep2	C8 Rep3
C_1	7	20.7	133.82	0.01	573.96	16.49	26.6	0.01	0.01	0.01	19.06	34.58	10.54
C_Benzoic	14	200.33	244.54	231.82	214.29	264.99	213.29	66.84	221.47	209.19	60.69	204.82	208.99
C_3	16	34.38	49.2	31.02	74.11	50.89	53.85	57.1	0.01	31.19	41.76	19.33	46.06
C_Vanillic	18	62.32	59.95	54.69	81.61	104.43	75.03	48.86	37.66	38.92	58.61	54.33	74.26
C_5	21	32.18	12.29	8.58	66.04	19.82	33.61	46.06	0.01	14.62	29.13	8.74	32.6
C_Syringic	22	43.52	41.09	30.21	42.49	60.06	45.54	27.53	25.53	19.27	36.82	38.24	48.86
C_7	30	90.73	97.31	114.08	69.67	88.66	81.05	70.04	66.6	73.96	35.56	44.93	32.58

Table A.1 Continued

Peak	App. RT	C5 Rep1	C5 Rep2	C5 Rep3	C6 Rep1	C6 Rep2	C6 Rep3	C7 Rep1	C7 Rep2	C7 Rep3	C8 Rep1	C8 Rep2	C8 Rep3
C_pCoum	33	306.41	353.99	297.68	258.89	400.76	223.96	217.22	271.12	214.03	176.17	192.9	240.45
C_9	38	568.04	684.77	778.86	621.37	959.42	489.1	544.79	664.59	623.95	380.72	532.92	504.32
C_Ferulic	39	2610.49	2679.85	2457.3	2260.25	2864.49	1968.99	2066.3	2240.03	1961.98	1850.53	2026.42	2444.3
C_11	57	271.85	296.23	281.77	151.73	68.92	40.05	178.06	173.85	157.93	0.01	0.01	0.01
C_11.5	57_	0.01	0.01	0.01	54.48	84.29	71.41	0.01	0.01	0.01	0.01	0.01	0.01
C_12	73.5	31.44	49.17	36.81	43.92	58.75	41.93	23.2	27.44	24.45	31.94	35.67	46.27
C_13	74.5	28.17	16.54	32.54	0.01	20.75	9.81	0.01	15.67	18.9	75.49	9.49	11.84
C_13.5	75.5	0.01	12.79	0.01	0.01	0.01	0.01	0.01	0.01	0.01	6.12	5.76	9.53
C_14	77	14.25	8.08	17.09	12.71	27.02	17.6	9.68	13.54	13.99	14.14	17.46	26.02
C_16	78	245.49	234.01	241.75	301.11	453.54	248.77	187.37	242.45	207.8	253.94	308.48	381.59
C_17	78.5	12.65	43.91	30.76	29.85	46.03	36.3	25.27	40.64	33.73	17.43	50.3	36.33
C_18	78.8	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
C_19	79	73.54	73.47	76.21	91.82	106.56	72.85	67.29	70.37	65.37	76.6	79.6	96.53
C_20	80	12	6.58	6.32	12.89	12.29	8.94	13.2	7.19	4.09	12.23	9.35	14.17
C_21	80.5	36.47	52.43	38.38	45.7	89.69	41.06	31.5	44.43	32.81	30.59	61.36	56.86
C_22	81.4	11.26	13.53	11.89	27.24	46.23	23.03	10.55	16.61	11.69	22.42	34.39	39.35
C_23	82	19.59	23.61	24.22	31.45	54.41	26.63	15.75	22.26	19.29	14.26	34.68	22.28
C_24	82.5	9.88	5.25	10.47	11.38	18.11	10.84	6.93	7.33	8.94	12.01	10.49	18.22
C_25	83	3.8	5.53	4.45	6.78	11.59	4.84	0.01	8.12	12.58	0.01	8.69	5.45
C_26	83.5	9.38	10.02	8.51	11.96	16.58	10.49	0.01	0.01	0.01	10.01	15.56	14.29

Table A.2: Retention times and mass fragments of mass spectra for secondary confirmation of identified compounds via mass spectrum.

Compound	Exact Mass	Standard		Free		Bound	
		RT (min)	m/z	RT (min)	m/z	RT	m/z
Protocatechuic	154.03	8.9	153	9.3	153	9.8	153
OH-Benzoic	138.03	17.9	137	14-19	137	18.5	137
Vanillic	168.04	28.6	167	29	167	28.9	167
Syringic	198.05	40.2	197	40.9	197	40.2	197
pCoumaric	164.05	50.6	163	51.3	163	51	163
Ferulic	194.06	64.7	193	68-71	193	65.1	193

Table A.3: Identified peak calibration curves with slope, y-intercept, standard error (SE) of the slope and y-intercept, R^2, F, sum of squares (SS) of regression residuals, and final LOD and LOQ in µg compound/g defatted tef flour. Vanillic acid standard eluted near chlorogenic acid and peaks were split via deconvolution in OriginPro (Northampton, MA 01060). Regression fit was done using the LINEST function in Microsoft Excel.

Protocat.			
Slope	25.02680917	0	y-intercept
SE Slope	0.74817268	---	SE intercept
R^2	0.989389369	18.80272266	SE Regression
Fisher's F	1118.94122	12	DF
SS Regression	395593.1414	4242.508554	SS Residuals
OH-Benzoic			
Slope	47.20216695	0	y-intercept
SE Slope	0.657652018	---	SE intercept
R^2	0.997289697	39.57441499	SE Regression
Fisher's F	5151.47455	14	DF
SS Regression	8067901.099	21925.8805	SS Residuals
Vanillic			
Slope	46.01029668	0	y-intercept
SE Slope	0.887601293	---	SE intercept
R^2	0.994816812	83.24712547	SE Regression
Fisher's F	2687.040513	14	DF
SS Regression	18621416.19	97021.17458	SS Residuals
Syringic			
Slope	49.84290443	0	y-intercept
SE Slope	0.962998658	---	SE intercept
R^2	0.99480113	79.13648364	SE Regression
Fisher's F	2678.892702	14	DF
SS Regression	16776788.01	87676.16261	SS Residuals
pCoumaric			
Slope	81.63364524	0	y-intercept
SE Slope	2.447623574	---	SE intercept
R^2	0.990208032	86.28288258	SE Regression
Fisher's F	1112.369634	11	DF
SS Regression	8281298.066	81892.09408	SS Residuals
Ferulic			
Slope	44.09026125	0	y-intercept
SE Slope	0.737654304	---	SE intercept
R^2	0.99443294	163.7366777	SE Regression
Fisher's F	3572.560427	20	DF
SS Regression	95779271.93	536193.9924	SS Residuals

ug/g	Free & Conj.		Bound	
	LOD	LOQ	LOD	LOQ
Protocat	1.7	5.14	1.7	5.14
Benzoic	1.89	5.74	1.89	5.74
Vanillic	4.09	12.38	4.09	12.38
Syringic	3.58	10.86	3.58	10.86
pCoumaric	2.39	7.23	2.39	7.23
Ferulic	8.39	25.41	8.39	25.41

Table A.4: Sample weights and volumes for extraction replicates for the free (A), conjugated (B), and bound (C) fractions.

Sample	Replicate	1 (TE_71)	2 (TE_73)	3 (TE_77)	1 (TE_71)	2 (TE_73)	3 (TE_77)
	Fraction	g DTF	g DTF	g DTF	Vol(mL)	Vol(mL)	Vol(mL)
DZ-409	A	1.88	---	1.87	1.2	---	1.2
DZ-409	A	1.87	1.95	1.91	1.2	1.4	1.4
DZ-409	A	1.88	1.93	1.98	1.1	1.3	1.3
DZ-01-974	A	1.88	1.92	1.89	1.1	1.4	1.3
DZ-01-974	A	1.86	1.94	1.96	1.4	1.4	1.3
DZ-01-974	A	1.90	1.92	1.93	1.2	1.5	1.4
DZ-Cr-37	A	1.88	1.96	1.98	1.4	1.4	1.4
DZ-Cr-37	A	1.87	1.99	1.81	1.2	1.4	1.3
DZ-Cr-37	B	1.88	1.93	1.87	1.0	1.4	1.4
DZ-01-196	B	1.87	1.95	1.91	1.4	1.5	1.4
DZ-01-196	B	1.88	1.93	1.98	1.5	1.4	1.5
DZ-01-196	B	1.88	1.92	1.89	1.4	1.5	1.5
DZ-Cr-384	B	1.86	1.94	1.96	1.5	1	1.3
DZ-Cr-384	B	1.90	1.92	1.93	1.4	1.4	1.3
DZ-Cr-384	B	1.88	1.96	1.98	1.4	1.4	1.4
DZ-01-99	B	1.87	1.99	1.81	1.3	1.4	1.4
DZ-01-99	C	1.88	1.93	1.87	1.3	1.4	1.4
DZ-01-99	C	1.87	1.95	1.91	1.3	1.4	1.4
TeffCoWhite	C	1.88	1.93	1.98	1.3	1.4	1.5
TeffCoWhite	C	1.88	1.92	---	1.4	1.4	---
TeffCoWhite	C	1.86	1.94	1.96	1.2	1.4	1.3
TeffCoBrown	C	1.90	1.92	1.93	1.2	1.0	1.4
TeffCoBrown	C	1.88	1.96	1.98	1.3	1.4	1.4
TeffCoBrown	C	1.87	1.99	1.81	1.3	1.4	1.4
Average weight: 1.91 g (Free/Conj) 0.25 g (Bound)					Average Volume: 1.3 mL		

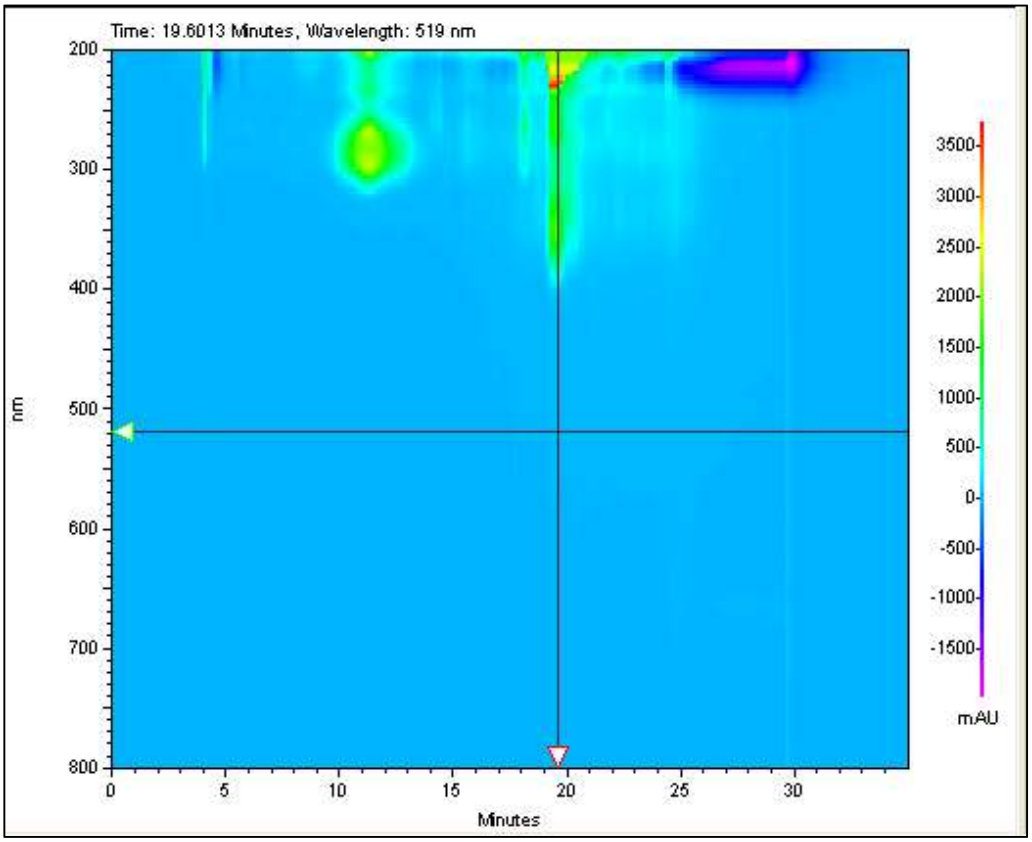


Figure A.1 Two-dimensional HPLC Chromatogram of extract from DZ-01-99. The horizontal line indicates 520 nm and no anthocyanin compounds were detected at this wavelength.

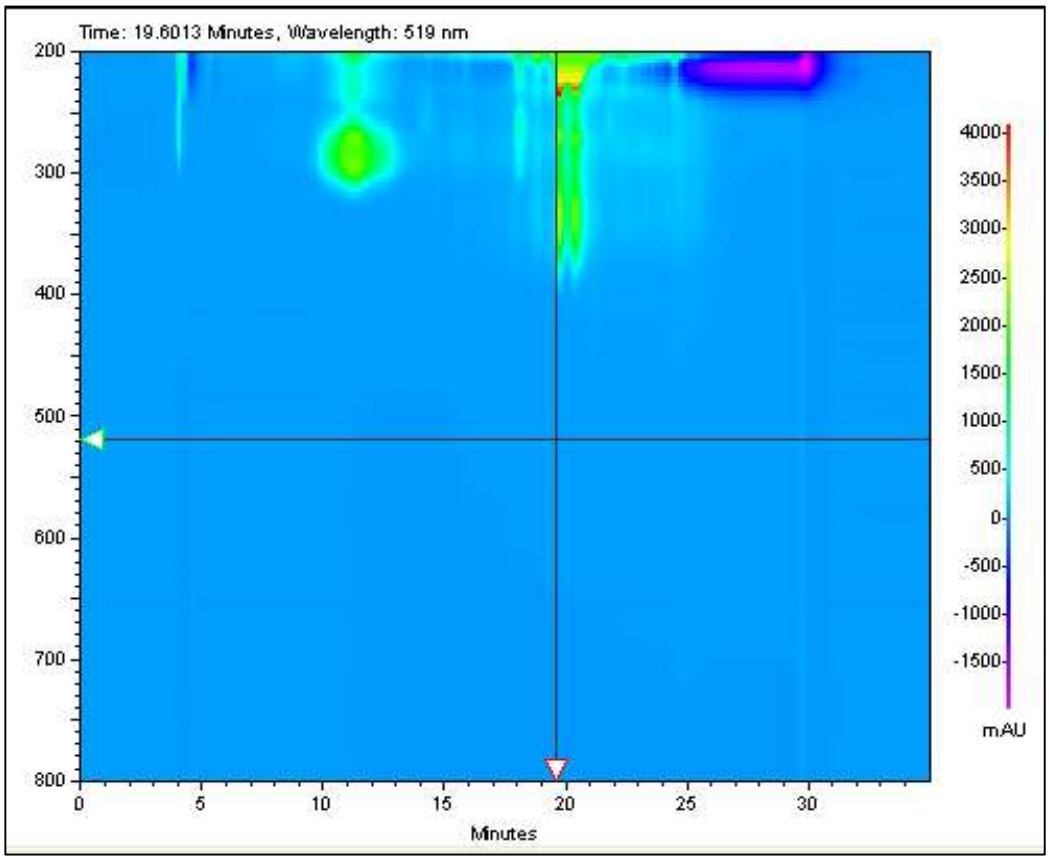


Figure A.2 Two-dimensional HPLC Chromatogram of extract from DZ-Cr-384. The horizontal line indicates 520 nm and no anthocyanin compounds were detected at this wavelength.

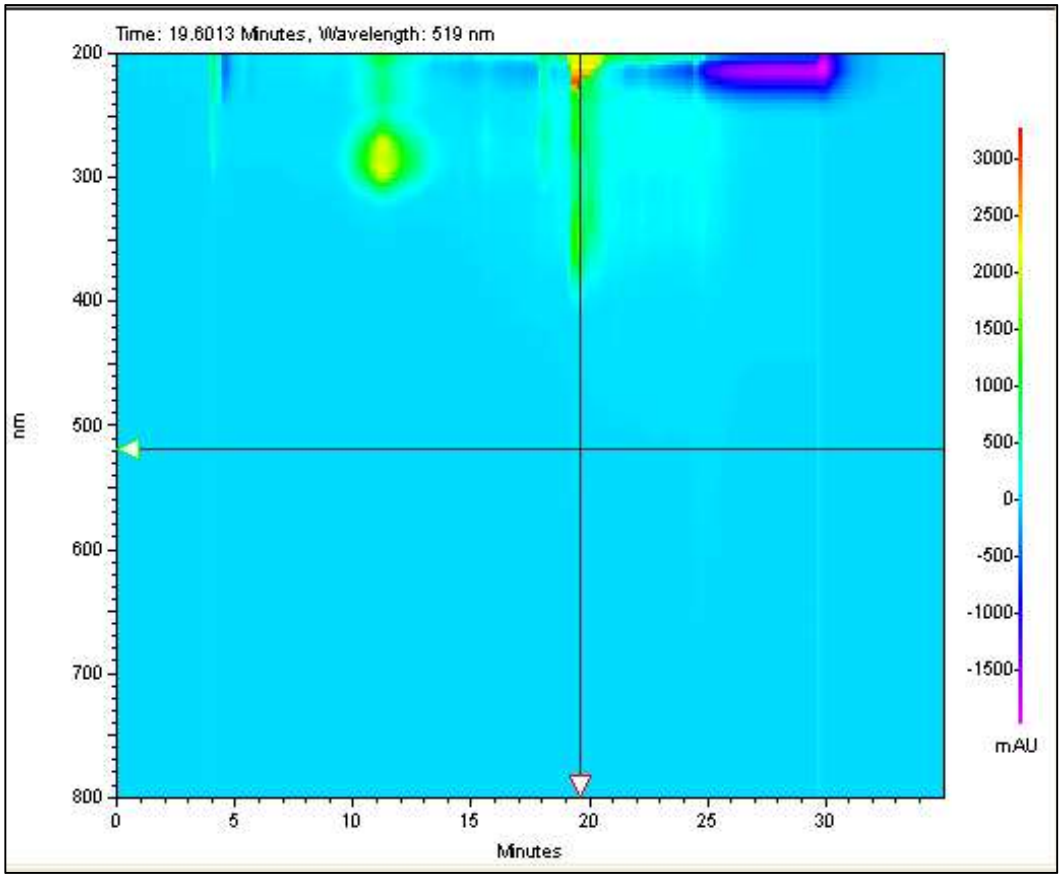


Figure A.3 Two-dimensional HPLC Chromatogram of extract from TeffCo Brown. The horizontal line indicates 520 nm and no anthocyanin compounds were detected at this wavelength.