INTERACTIVE EFFECTS OF CEREAL-PULSE FLAVONOID COMBINATIONS ON THEIR BIOAVAILABILITY

A Dissertation

by

SHREEYA RAVISANKAR

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Chair of Committee,	Joseph M. Awika
Committee Members,	Chaodong Wu
	Bhimanagouda Patil
	Peter Murano
Head of Department,	Steve Searcy

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ABSTRACT

Cereals and pulses are consumed together in several cultures. They contain complementary flavonoid structures, that, in a previous study, showed synergistic downregulation of inflammation in CCD-18Co cells. However, the mechanisms of the observed effect are unknown. Further, there are gaps in knowledge on flavonoid profiles in cereals and effect of processing on the flavonoids.

The goal of this study was to evaluate the interactive effects of cereal-pulse flavonoid combinations on their bioavailability, which is an important determinant of bioactivity. The absorption properties of flavonoid combinations were determined in Caco-2 monolayer model. Structural variation of flavonoids in different cereal grains was evaluated using ultra-performance-liquid-chromatrography-tandem-quadrupole-mass-spectrometry. Lastly, effect of fermentation and baking (pancakes) on different flavonoid structures in grains was evaluated.

Apparent absorption of quercetin and apigenin (representing flavonols-flavones predominant in pulses-cereals) increased 3.3 and 1.5 times respectively, and they metabolized to a lesser extent in combined treatments vs individual treatments. Combinations with naringenin (flavanone present in cereals) had an insignificant effect, suggesting a role for flavonoid C2=C3 double bond conjugation. Further, apigenin-quercetin combinations inhibited P-glycoprotein ATPase activity by 40-66% suggesting direct binding to ABC transporters. Thus, cereal-pulse flavonoid combinations showed enhanced bioavailability due to reduced metabolism and synergistic downregulation of ABC transporter function.

Profiling of different cereal grains showed wheat mainly contained di-C-linked-glycosyl flavones. Rye and teff grains contained C-linked (di/mono/O-glycosyl-C-glycosyl-flavones), O-glycosyl flavones and phenolic acids (9 compounds identified for the first time in rye, 32 compounds identified for the first time in teff). Sorghum mainly contained aglycones of flavones, O-glycosyl flavanones and phenolic acids. Fermentation of grains completely metabolized phenolic acids to reduced and decarboxylated derivatives; hydrolyzed 25-100% of flavonoid-O-glycosides to their aglycones; released bound phenolic acids; but had no effect on C-linked flavonoids (mono-C-linked/di-C-linked flavonoids). Further, antioxidant activity increased with fermentation of dough (1.7 – 3.4 times vs non-fermented dough) and further increased in fermented pancakes compared to corresponding doughs (1.2 – 1.7 times). Thus, cereals show structural variations in their polyphenols that can be manipulated through processing methods to influence potential bioavailability of the polyphenols.

Overall, strategic combinations of cereals and pulses accompanied by appropriate processing methods could enhance the bioavailability of flavonoids from whole grain products.

DEDICATION

To my Dad, Ravisankar Swaminathan and Mom, Shanthini Ravisankar

I love you both.

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Contributors

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NOMENCLATURE

ROS	Reactive oxygen species
RNS	Reactive nitrogen species
TNF-α	Tumor necrosis factor alpha
IL-1β	Interleukin – 1 beta
IL-6	Interleukin – 6
IFN-γ	Interferon gamma
IL-8	Interleukin – 8
MCP-1	Monocyte chemoattractant protein – 1
COX-2	Cyclooxygenase-2
MAPK	Mitogen-activated protein kinases
ERK	Extracellular signal-regulated kinase
JNK	c-Jun N-terminal kinase
NF-κB	Nuclear factor kappa-B
CVD	Cardiovascular disease
ER-β	Estrogen receptor beta
NO	Nitric oxide
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharides
CYP1A1	Cytochrome P450 Family 1 Subfamily A Member 1
PGE-2	Prostaglandin E2
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

DPPH	2,2-diphenyl-1-picrylhydrazyl
ORAC	Oxygen radical absorbance capacity
TEAC	Trolox equivalent antioxidant capacity
EPC	Extractable phenol content
ABC transporters	ATP-binding cassette transporters
ATP	Adenosine tri phosphate
ADP	Adenosine di phosphate
LPH	Lactase phlorizin hydrolase
SGLT-1	Sodium glucose co-transporter 1
UGT	Uridine 5'-diphospho-glucuronosyltransferase
SULT	Sulfotransferase
COMT	Catechol-O-Methyl transferase
BCRP	Breast cancer resistance protein
MRP	Multidrug resistance associated protein
P-gp (MDR1)	P-glycoprotein (Multidrug resistance mutation 1)
UPLC	Ultra performance liquid chromatography
ESI	Electrospray ionization

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1. INTRODUCTION

Chronic diseases such as cardiovascular disease, cancer, and diabetes are a leading cause of death in developed countries and a growing burden in developing countries [1-2]. Poor dietary choices and lack of physical activity among other factors can lead to obesity and chronic inflammation, which has been implicated as a major driver of chronic diseases [3]. Polyphenols, especially the flavonoid group of compounds, are gaining increased attention as a nutrition-based strategy to mitigate chronic inflammation. A large body of evidence indicates these compounds to have strong potential to inhibit several inflammatory and chronic disease targets [4-6].

Cereal grains and grain pulses are staple foods around the world, contributing a major proportion of human caloric intake. Several epidemiological studies have shown that consumption of whole grain cereals and pulses is strongly associated with reduced risk of chronic diseases [7-10]. Cereals and pulses are traditionally consumed together in many cultures, and provide complementary amino acids that helps overcome protein deficiency [11-12]. Interestingly, cereals and pulses also contain structurally different flavonoid classes [13]. For example, the most commonly consumed cereals (wheat, barley, sorghum, oats, millets) are abundant in flavonoids that are unsubstituted at the C-3 position of the flavonoid heterocyclic ring (flavones, flavanones, 3-deoxyanthocyanins). On the other hand, commonly consumed pulses such as common beans, cowpea, chickpea, peas, and lentils contain mainly 3-hydroxy flavonoids (flavonols, flavanols, anthocyanins) [14]. This difference in flavonoid structure could likely complement each other for enhanced bioactive properties. Based on this hypothesis, a recent study in our lab showed that

combination of sorghum flavones and cowpea flavonols as well as pure compounds apigenin and quercetin (the most abundant flavone and flavonol type in sorghum and cowpea) were strongly synergistic in downregulating the inflammatory transcription factor NF-κB and its related downstream cytokines [15]. However, the mechanisms for the observed strong synergistic action are not well understood.

In order for the ingested flavonoids to show bioactive properties against chronic diseases, they need to be bioavailable from the food consumed. Two major factors limiting the bioavailability of flavonoids from the small intestine are: 1) metabolism by phase 2 enzymes that are involved in transforming flavonoids to more hydrophilic forms (glucuronides, sulfates) that are excreted through urine, and 2) ATP binding cassette (ABC) transporter proteins that are energy dependent efflux pumps located in the apical membrane of intestinal cells responsible for eliminating their substrates back into the lumen for excretion [16-19]. Several studies have shown that, depending on their structure, flavonoids and their metabolites might act as inducers or inhibitors of ABC transporters as well as phase 2 metabolizing enzymes[17, 20-22]. Based on this, we hypothesize that, the anti-inflammatory synergy of combined cereal-pulse flavonoids is due to their enhanced bioavailability via synergistic downregulation of ABC membrane transporter and metabolizing enzyme activity.

Because the structure of flavonoids as present in cereals and pulses is an important determinant of their absorption as well as bioactive properties, it is important to accurately profile flavonoids in different grains using advanced techniques. While pulses have been well profiled for flavonoids, literature reveals a gap in knowledge on the structural

variation in flavonoids in cereals. A thorough knowledge on the various flavonoid structures present in the grains as well as their quantity is important to validate the potential health benefits of the individual commodities as well as establish the interactive effects between different commodities.

Several processing strategies are commonly employed before the consumption of grains. These include milling, cooking, extrusion, and bioprocessing techniques such as germination and fermentation. Among these techniques, fermentation not only helps in improving the overall nutritional value, shelf life, and sensory properties of whole grain products [23-24], but has also been shown to improve the bioaccessibility and bioavailability of polyphenols [25]. However, there are no studies evaluating the effects of fermentation and subsequent heat treatment on flavonoids, especially di-C-linked-glycosides and aglycone forms in cereal grains. Understanding the effect of fermentation on different flavonoid structures could have important implications in developing processing technologies that enhance benefits of flavonoids upon consumption of fermented products made from whole grains.

The overall goal of this study was to evaluate the interactive effects of cereal and pulse flavonoids on their bioavailability. The hypothesis is that structurally different flavonoids as present in abundance in cereals (3-deoxyflavonoids) and pulses (3-hydroxyflavonoids), could likely show enhanced bioavailability in combination. Since the structural features of flavonoids dictates their functionality, different grains commonly consumed were profiled for the flavonoids in them. Further, the effect of fermentation on different flavonoid structures was investigated, as this is a unique processing technique

that could influence the bioavailability of flavonoids. Outcomes of the work could help strategically combine cereals and pulses to develop novel functional food formulations offering enhanced health benefits.

The specific objectives of this work include:

1. To determine mechanisms of synergistic anti-inflammatory action of cereal-pulse flavonoid combinations in Caco-2 model
2. To determine the variations in structure and profile of flavonoids in cereal grains
3. To determine effect of fermentation and baking on cereal grain flavonoids and their antioxidant properties

2. LITERATURE REVIEW

2.1. Prevalence of chronic diseases

The prevalence of non-communicable chronic diseases in the United States is continuously on the rise and is projected to affect approximately 48% of the US population by 2020 [1]. A report on the global burden of disease (2015), also shows the trend in increase in prevalence of non-communicable diseases globally over communicable diseases, with vascular related diseases being the most prevalent [26]. Ischemic heart disease and stroke caused about 15.2 million deaths in 2015 [26]. Different forms of cancer caused 9.6 million deaths worldwide in 2018 [27]. On the other hand, estimates suggest that diabetes affected 415 million people (ages 20 – 79 years) in 2015 and this number is likely to increase to 642 million in 2040 [28]. Thus, there is a major need to control the development and progression of chronic diseases globally.

There are certain common risk factors associated with the development of chronic diseases. They include lack of physical activity, high body mass index (BMI) and poor nutrition (diets low in fruits, vegetables and whole grains and high in sodium and saturated fats) which can lead to obesity and associated conditions [1, 29-30]. Obesity, along with the other risk factors, over time, lead to the development of chronic inflammation, which has been implicated as a major driver of several chronic diseases [31-32].

2.2. Inflammation and link to chronic diseases

Inflammation is the body's protective mechanism and normal biological process in response to several triggers such as tissue injury, microbial infection, chemical irritation

and other endogenous (reactive oxygen species (ROS), reactive nitrogen species (RNS), leucocytes) and exogenous factors (e.g. UV or ionizing radiation, pollutants, allergens) [4, 32]. The process involves immune cells releasing several inflammatory mediators such as ROS, RNS, inflammatory cytokines (TNF-α, IL-1β, IL-6, IFN-γ), chemokines (IL-8, MCP-1) and eicosanoids (prostaglandin E2, leukotrienes – mediated by COX-2 enzyme) [32-33]. Normally these mediators play a role in host defense and clearance of infection, and inflammation is a self-limiting process (acute inflammation). However, continuous exposure to risk factors leads to development of chronic inflammation wherein the body lacks a balance between the inflammatory trigger and inflammatory response causing more damage to the host than to resolve the trigger. This could lead to tissue destruction, fibrosis and development of various chronic diseases like cancer, cardiovascular disease and type 2 diabetes. Chronic and low-grade inflammation is also responsible for "inflammageing" responsible for development of type-2 diabetes, atherosclerosis, cancer, loss of mobility, immune decline in the elderly [34]. The underlying mechanism involves the inflammatory mediators targeting certain signal transduction pathways (MAPK - ERK, JNK, p38 and hence NF-κB pathway). These signal transduction pathways are key regulators controlling cell growth, proliferation, apoptosis and release of pro-inflammatory cytokines and chemokines that can interfere with proteins/receptors governing insulin resistance, cancer or cardiovascular disease [4, 33]. Thus, chronic inflammation is an underlying cause of several chronic diseases.

2.3. Whole grains and chronic disease prevention

Whole grain cereals and pulses are a staple food and a major source of calories for humans around the world. Several epidemiological studies have shown their consumption to be strongly associated with reduced risk of inflammation related chronic diseases [35-37]. For example, there was lower risk of total mortality (17%) and CVD associated mortality (9%) in US men and women consuming diet rich in whole grains independent of other dietary and lifestyle factors [38]. High dietary intake of both whole grain and whole grain products was reported to protect against esophageal cancer in Scandinavian countries [39]. On the other hand, intake of legumes more than 4 times a week vs once a week has been associated with a decreased risk of coronary heart disease (by 22%) and CVD (by 11%) [40]. A review further suggested that combination of the myriad of bioactive compounds that whole grains offer rather than a single component (e.g. fiber) is the reason for their protective CVD action [41].

Whole grain cereals and grain pulses are traditionally consumed together in many cultures around the world (Africa, Asia, United States, Latin America and Middle east) and this combination helps overcome protein deficiency through complementary amino acid profile [12, 42-43]. While cereals have a low content of lysine and a high content of methionine, pulses provide adequate lysine but are low in methionine [11-12]. Interestingly, recent studies are showing that benefits offered by these commodities may not only be limited to protein or fiber, but may also be due to synergistic interactive effects between the different classes of polyphenols present in cereals and pulses [13, 15, 44-45]. This suggests

that strategic combination of diets with whole grain cereals and pulses may provide complementary health benefits beyond protein nutrition.

2.4. Structural differences of polyphenols in cereals and pulses

Polyphenols are secondary plant metabolites with more than 8000 structures being recognized. Out of this, almost 6500 structures belong to the class of flavonoids. A large body of evidence suggests that flavonoids have strong potential to inhibit several inflammatory and chronic disease targets [4]. Flavonoids are compounds with a C6-C3-C6 backbone structure (Fig 1) and are classified based on hydroxylation pattern and variations in the heterocyclic ring. The major classes of flavonoids generally identified in cereals and pulses include flavonols, flavones, flavanones, anthocyanins, flavan-3-ols and proanthocyanidins [14].

The majority of cereals, such as wheat, barley, sorghum, oats, and millets such as pearl, fonio and foxtail millet mainly contain 3-deoxyflavonoids (e.g. flavones (apigenin, luteolin), flavanones (naringenin, eriodictyol), 3-deoxyanthocyanins). Most flavonoids in grains are conjugated with one or more sugar residues (e.g. glucose, galactose) linked to hydroxyl groups (O-linkage) or directly linked to an aromatic carbon atom (C-linkage) on the flavonoid molecule [46]. Among the cereals, differences exist in flavonoid structures both with respect to flavonoid classes as well as type of sugar conjugation. A vast body of literature suggests that most of the commonly consumed cereals such as wheat, barley, pearl millet, oats mainly contain C-linked flavone glycosides (mainly apigenin-C-glycosides) (Fig 1) [47-49]. Sorghum is unique in that different varieties mainly contain the

aglycone form of 3-deoxyflavonoids (flavones, flavanones) or their respective O-glucoside forms [50].

Cereals are the most important source of flavones in human diet, as other major sources of dietary flavones including celery, herbs like parsley or chamomile flowers are consumed in much lower abundance and infrequently as part of daily diet compared to cereals [51-53]. This unique abundance of flavones in cereals is extremely important owing to their structural features (C2=C3 double bond conjugation and lack of C3-hydroxyl group substitution) that are known to contribute to strong anti-inflammatory action [15]. Further, flavones have also been shown to protect against estrogen-linked colon carcinogenesis [54]. Flavones also recently showed strong synergistic anti-inflammatory action in combination with pulse derived flavonols [15].

Though cereals have been widely profiled for phenolic acids, the profile and content of flavonoids in the different cereal grains is still poorly understood, providing little structural information and/or reliable quantitative data. For example, the differences in flavonoids between red and white wheat likely leading to their differences in pigmentation [55] or reliable quantitative information of flavonoids in grain rye [56] are still lacking. Further, discrepancies exist in literature regarding the type of identified flavonoids in different grains. For example, certain studies with grain teff report flavanone (naringenin, eriodictyol) and flavan-3-ol catechin to be the most abundant in white and brown teff [57] while others report flavones to be the most abundant [58] with no distinction between the different colored grains. These gaps in literature needs to be improved upon

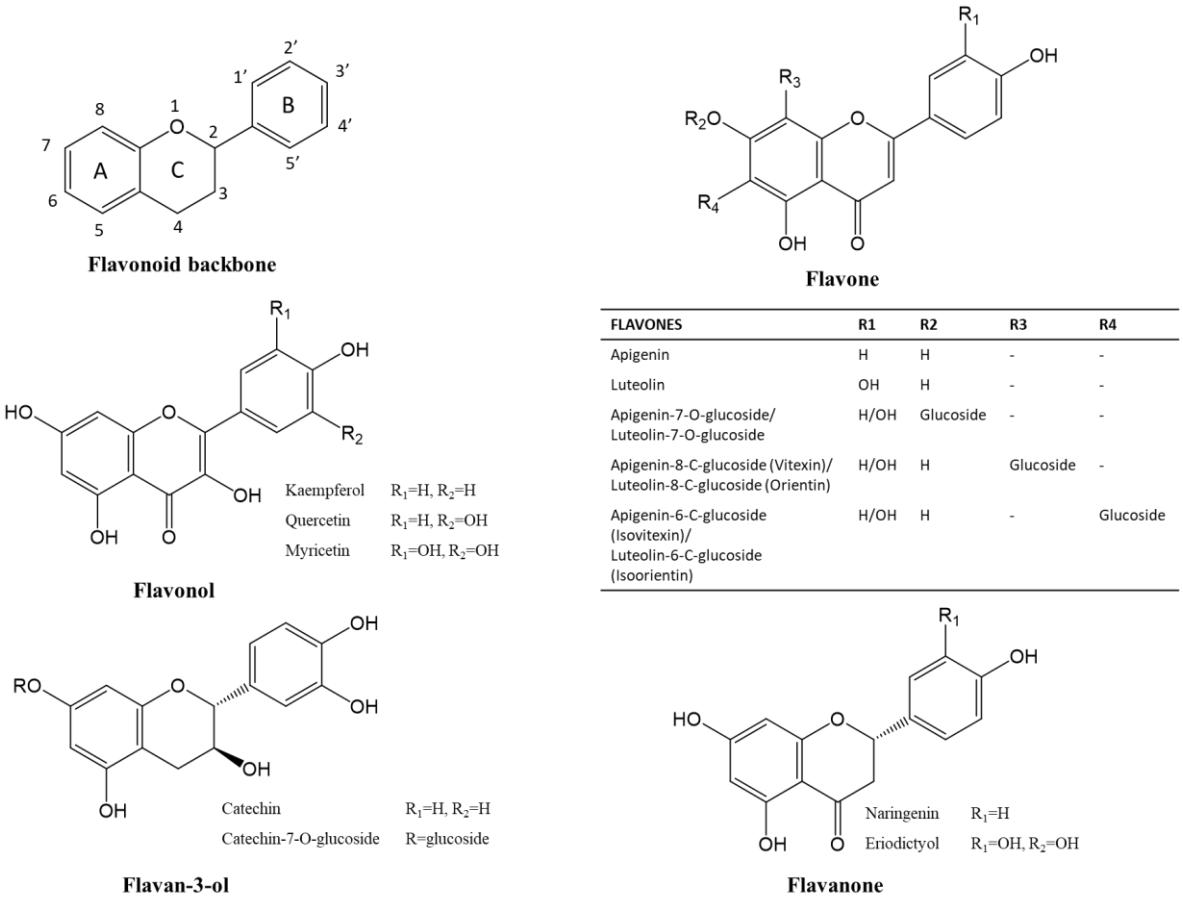
for not only understanding the potential health benefits of the individual grain commodities, but also for their potential interactive effects with pulse flavonoids.

Unlike cereal grains that mostly contain the 3-deoxyflavonoids, grain pulses such as common beans, cowpea, chickpea and lentils, mainly contain 3-hydroxyflavonoids (e.g. flavonols, flavan-3-ols, anthocyanins). Amongst the flavonols, quercetin, kaempferol or myricetin conjugates are the most widely distributed forms in pulses and are mainly O-linked to sugars [59-62]. Further, certain varieties of beans, cowpea and lentils also contain significant levels of flavan-3-ols, especially monomeric catechin or epi-catechin and their respective O-glucosides [63-65]. Anthocyanins are also found in black and green cowpea varieties and in black and red kidney beans [59-60].

The class of polymeric flavanols (proanthocyanidins/condensed tannins) are present in significant quantities only in certain grains like sorghum [66]. They have generally been less studied for their anti-inflammatory properties as their large molecular weight prevents permeation through the cell membrane to influence intracellular inflammatory pathways [14]. Thus, benefits associated with proanthocyanidins could be mostly indirect, either through their interaction and metabolism by gut microbiota, or potential to complex with macromolecules and slow digestion of starch (a major source of carbohydrate derived calories for humans) [14, 67]

Overall, it can be deduced that commonly consumed cereals and pulses contain structurally different classes of flavonoids, with flavone class of compounds being more widespread in cereals and flavonol class of compounds being more widespread amongst

pulses. These structural differences have the potential to synergistically enhance bioactive properties of combined cereal-pulse intake [13].



FLAVONES	R1	R2	R3	R4
Apigenin	H	H	-	-
Luteolin	OH	H	-	-
Apigenin-7-O-glucoside/ Luteolin-7-O-glucoside	H/OH	Glucoside	-	-
Apigenin-8-C-glucoside (Vitexin)/ Luteolin-8-C-glucoside (Orientin)	H/OH	H	Glucoside	-
Apigenin-6-C-glucoside (Isovitexin)/ Luteolin-6-C-glucoside (Isoorientin)	H/OH	H	-	Glucoside

Figure 1: Flavonoid backbone along with common flavonoid structures identified in different cereals (flavone, flavanone) and pulses (flavonol, flavan-3-ols).

2.5. Effects of fermentation on flavonoids in grains

Whole grain cereals and pulses are processed in several ways before consumption. Common techniques involve various physical processes such as milling and grinding to

produce flour and thermal processes such as open pan boiling, steaming, cooking, roasting, baking, extrusion and bioprocessing techniques such as germination and fermentation.

Amongst these processes, fermentation provides increased functionality in products, such as extending shelf life and improving the flavor and nutritive value of foods [23-24]. Traditionally grains are fermented in several cultures around the world. For example, teff is traditionally consumed as a fermented pancake like product, *Injera*, in Ethiopia [68], wheat and rye in (sourdough) bread [69] and several pulses are fermented into snack or breakfast products in countries like India, Indonesia, Sri Lanka, Nigeria and in regions in West and Central Africa [23]. Fermentation is also gaining popularity in gluten free applications owing to added advantages of improving texture due to release of microbial exopolysaccharides replacing the use of gums [70-71]. Also, fermentation has been found to reduce the toxicity of wheat glutens in celiac patients due to partial hydrolysis of the gliadin peptides that are responsible for the autoimmune response [70].

Fermentation is carried out with bacteria, fungi and/or yeast releasing several enzymes that can not only release bound phenolics (due to esterase activity) but also induce structural changes to flavonoids, especially the glycoside forms (due to glucosidase activity). This could directly affect the bioaccessibility, bioavailability and bioactivity from fermented products [72]. For example, in a sourdough, fermented from red sorghum, there was increase in levels of aglycone naringenin and eriodictyol which corresponded to a decrease in O-glucoside forms of the same, likely indicating glucosidase activity by lactic acid bacteria [25]. Further, there was significant degradation of phenolic acids and their esters being converted to corresponding metabolites (e.g. dihydroferulic acid,

vinylcatechol, ethylcatechol, dihydrocaffeic acid) due to phenolic acid decarboxylase and reductase activity [25]. In line with this, fermentation of pulse, cowpea, with *Lactobacillus plantarum* also showed reduction in levels of phenolic acids ferulic, p-coumaric acid and hydroxycinnamic acid derivatives, with an increase in levels of aglycone quercetin, due to hydrolysis of quercetin-O-glucosides [73].

One of the few studies evaluating effect of fermentation on C-glycosylflavones, vitexin and isovitexin, in mung bean milk, did not show changes in structure or concentration of the flavonoids upon fermentation [74]. Thus, there could be differential effects of fermentation on flavonoids based on their conjugation type to sugars (O- or C-linkage). However, there are no studies evaluating the effect of fermentation and subsequent thermal treatment on the C-linkedflavones (di-C-linked, mono-C-linked and C-and-O-linked glycosides) as present in grains such as wheat, with most of these studies only looking at profile of phenolic acids. Thus, it is important to study the effect of fermentation on flavonoids in grains as it could have key implications on flavonoid extractability and bioaccessibility, bioavailability, and bioactivity upon consumption of fermented products based on these grains.

2.6. Properties of cereal and pulse flavonoids against inflammation

As indicated in the previous section, the most commonly consumed cereals contain mostly flavones (O and C-linked glycosides) or flavanones, whereas pulses mainly contain flavonols and flavan-3-ols. These structural differences could have major implications on the inflammatory action of cereal and pulse flavonoids. For example, a study showed that apigenin was able to downregulate inflammation via the NF-kB pathway in human

placental and fetal membrane tissues at a much lower concentration (5 μM) compared to naringenin (200 μM) [75]. A study from our lab also showed that apigenin was 10 times more effective than naringenin in activating ER-β receptor in non-malignant colonocytes thus showing better protective action against colon cancer [76]. These studies indicate that flavonoids with C2-C3 double bond (as present in apigenin (flavone)) show stronger inhibition of chronic disease related targets. This property is likely due to stronger binding to the respective protein targets by the flavonoids with a C2-C3 double bond than flavonoids lacking the C2-C3 double bond (e.g. naringenin (flavanone)) [14, 77]. The C2-C3 conjugation makes the molecule relatively planar as it removes the chirality at C2 (which would twist the B-ring relative to the A&C rings), likely aiding in better interaction with protein targets [14].

Further, C-glycosyl flavones show differences in their action against diabetic, Alzheimer's and inflammatory targets, with vitexin and isovitexin showing enhanced action against diabetic (RLAR, HRAR, PTP_1B & AGE) and Alzheimer's targets (AChE, BChE and BACE enzymes), whereas apigenin showed enhanced protection against inflammatory targets NO, iNOS and COX-2 enzymes [78]. Similarly, luteolin showed enhanced protection against diabetic, Alzheimer's and inflammatory targets compared to its C-glycoside forms [79]. The difference in action was hypothesized to be due to additional electron density being drawn towards the A-ring in C-glycosylflavones which could affect their inhibition potential of various enzymes [78]. Thus, nature of glycosylation could have differential effects on flavonoid interaction with chronic disease targets.

On the other hand, a study from our lab evaluated different cowpea varieties on their anti-inflammatory action in RAW 264.7 macrophages (inflammation induced using LPS) [80]. The authors found that, white cowpea containing exclusively flavonol derivatives (quercetin-O-glucosides) downregulated NF-kB expression, whereas, a red cowpea variety containing both flavan-3-ol (catechin-O-glucoside) and flavonols (quercetin-O-glycosides), upregulated the expression of NF-kB [80]. Another study showed that quercetin was a 15 times stronger inducer of CYP1A1 mRNA expression compared to taxifolin (flavanonol – a quercetin analog without C2-C3 double bond conjugation). The authors also found that quercetin showed stronger potential to activate AhR in HepG2 cells, thus showing potentially enhanced protection against cancer compared to taxifolin [81]. These studies further indicate that, along with the C2-C3 double bond conjugation, the presence of 4-oxo group (as present in quercetin) enhances the potential interaction of these compounds with chronic disease targets as compared to flavonoids lacking these features (e.g. catechin) (Fig 1).

How these structural features of the different flavonoids as present in cereals and pulses could translate to their interactive bioactive effects when consumed in combination are largely unknown.

2.7. Cereal-pulse flavonoid combinations against inflammation

Few studies have evaluated flavonoid combinations as relevant to cereals and pulses and their effect on inflammation. A study evaluating different combinations of flavonols, flavones and flavanones showed that flavonol-flavone combination had strongest synergy in downregulating inflammatory markers TNF-α, nitic oxide and PGE-2 compared to

other combinations [82]. Apea-Bah et al. found that a sorghum-cowpea composite porridge (flavone-flavonol) showed synergistic potential to alleviate markers of oxidative stress *in vitro* (ABTS, DPPH, NO scavenging activity) compared to a maize-soybean (flavanone-isoflavone) composite porridge [44].

A recent study in our lab showed that combination of white sorghum (abundant in flavone-apigenin) with white cowpea (abundant in flavonol, quercetin-O-glucosides) extracts showed synergistic antioxidant effect, exhibiting 2.1 times higher ORAC value compared to additive effect [13]. On the other hand, the white sorghum extract combination with brown cowpea extract (abundant in catechin-O-glucoside and flavonols (quercetin derivatives)) only showed additive effect [13]. In line with these studies, white sorghum flavones and white cowpea flavonols showed strong synergistic downregulation of inflammatory targets in CCD-18Co colon myofibroblasts, with IC_{50} for additive effect being 8.3-21 times higher for downregulating expression of LPS-induced NF-κB and related downstream targets. Similar effects were also observed with pure apigenin-quercetin combinations, confirming that the observed effects were due to flavone-flavonol combinations [15]. Thus, cereal-pulse flavonoid combinations with structures governed by a flavone-flavonol interaction, show strong synergistic potential against markers of inflammation. This effect may be partly due to enhanced non-competitive binding affinity with molecular protein targets and receptors, though this has not been demonstrated.

In all, these studies reiterate the fact that the C2-C3 double bond conjugation and 4-oxo group play a significant role even in anti-inflammatory synergistic interactions among flavonoids. Further, the differences in C-3 substitution (flavonol vs flavone) could also

affect the nature of their interactions with anti-inflammatory targets in a synergistic manner. A flavonoid with C2-C3 double bond with C3 hydroxyl group (flavonols) is completely planar. Whereas flavonoids without the C3 hydroxyl (flavone) have a slightly twisted B-ring by 20° relative to the rest of the molecule [83]. The C3 substitution also makes flavonols more redox reactive than a flavone due to lower dissociation energy of the hydroxyl group [83-84]. Thus, a combination of a flavonol with a flavone could show sustained protective action against oxidative stress due to regeneration of the flavonol by the flavone.

Although studies have shown the synergistic effects of flavone-flavonol combinations against inflammation, there is lack of knowledge on the possible mechanisms governing the synergy. A likely mechanism leading to enhanced anti-inflammatory effects of flavonoids in combination could be related to their bioavailability and factors governing their bioavailability: phase 2 metabolizing enzymes and ABC transporters. Further, although knowledge from literature has contributed to understanding interactive potential of different flavonoids, it is also important to study the interactive effects of flavone-C-glycosides (as found in cereals) with pulse flavonoids. Evidence suggests that the C-glycosides are absorbed and metabolized differently as compared to aglycones or O-glycoside forms [6].

2.8. Bioavailability of flavonoids

In order for the ingested flavonoids to show bioactive potential against chronic diseases, they need to be bioavailable from the food consumed. Bioavailability is defined as the fraction of ingested nutrient or compound that reaches systemic circulation and is

available for physiological function and/or storage [85-86]. The process of bioavailability involves the following: 1. Bioaccessibility of polyphenols from the food matrix 2. Changes in polyphenols during gastrointestinal digestion 3. Cellular uptake of conjugated or aglycone form of compounds by enterocytes of the small intestine 4. Metabolism of polyphenols which involves enzymatic modification in the small intestine/liver (mainly phase 2 metabolism) 5. Efflux or uptake by ATP binding cassette transporters 6. Systemic circulation and tissue distribution 7. Excretion via kidney or re-excretion into gut via bile 8. Microbiological fermentation to metabolites of non-absorbed polyphenols as well as those re-excreted via bile/pancreas [85].

2.8.1. Differences in absorption properties of flavonoid-O-glycosides and flavonoid-C-glycosides

Upon ingestion, flavonoids are typically released from the food matrix via digestion in the stomach (pepsin digestion along with peristaltic movements and low pH) which causes disruption of plant cell wall and cellular components, reduction in particle size of foods, thus allowing for enhanced enzyme access to break down food macromolecules. Further, the low pH in the stomach favors the diffusion of flavonoids from food matrix into the aqueous phase due to reduced ionic interactions [85]. Flavonoids typically do not undergo significant changes in their structure from the oral cavity until the stomach where they are majorly released.

Upon reaching the small intestine, most of the O-linked flavonoid mono glycosides are hydrolyzed to their aglycone form by brush border enzymes (e.g. lactase phlorizin hydrolase, LPH) [17, 51, 85, 87]. The substrate specificity for LPH varies amongst glucosides,

galactosides, arabinosides, xylosides and rhamnosides, with the most efficient cleavage being of glucosides irrespective of the flavonoid [88]. After cleavage, the aglycone forms are then absorbed by passive diffusion and/or facilitated/active transport through the enterocytes of the small intestine [85]. Certain flavonoid glucosides are also absorbed by sodium-glucose cotransporter 1 (SGLT1) present in the small intestine (e.g.: quercetin-3-glucoside or quercetin-4'-O-glucoside) and then cleaved by cytosolic β-glucosidase present in the enterocytes to their corresponding aglycone forms [89-91]. Interestingly, flavonoid-O-glucosides, are absorbed to a greater extent compared to their aglycone form in the small intestine wherein a study reported that quercetin-3-O-β-glucoside showed 3 times more absorption potential in plasma compared to quercetin aglycone in rats (the glucoside was absorbed after hydrolysis to aglycone, and no O-glucoside forms were detected in the plasma; all forms identified in both quercetin-3-O-β-glucoside group and quercetin group were corresponding metabolite forms) [92].

On the other hand, flavonoid-C-glycosides typically resist hydrolysis in the small intestine, and they are also not substrates for SGLT-1 transporters. They thus show limited absorption in the small intestine with most of them reaching the colon [5]. A study showed that, apparent permeability co-efficient (which estimates the absorption potential into systemic circulation from the small intestine) for vitexin (apigenin-C-glucoside) was 2.26 ± 0.37 × 10^{-6} cm/s and for orientin (luteolin-C-glucoside) was 0.75 ± 0.05 ×10^{-6} cm/s; whereas it was much higher for the respective aglycone forms apigenin (10.9 ± 0.9 × 10^{-6} cm/s) and luteolin (8.87 ± 0.63 × 10^{-6} cm/s) [93-94].

The flavonoids showing lower absorption potential in the small intestine reach the colon, where they can be deglycosylated and absorbed or further transformed into colonic metabolites. However, the absorption potential of flavonoids from colon is much reduced compared to the small intestine. For example, an *in vivo* study comparing the absorption of quercetin aglycone, quercetin-3-O-glucoside and quercetin-3-O-rutinoside showed that quercetin-3-O-glucoside was efficiently deglycosylated and rapidly (t_{max} < 0.5h) absorbed in plasma. On the other hand, quercetin-3-O-rutinoside that resisted deglycosylation in the small intestine, reached the colon where it was deglycosylated. However, the deglycosylated form only showed 20% of the absorption potential of the 3-O-glucoside (t_{max} approx. 6h) from the colon [95]. Similarly, studies have shown that, C-linked mono glycosides typically reach the colon, where they can be deglycosylated, and absorbed or further undergo ring fission to produce colonic metabolites such as phloroglucinol, hydrocaffeic acid and phloretic acid [96]. Such metabolites may show different biological properties vs the intact flavonoids. Further, studies show that < 1% of flavone-C-glycosides were excreted through urine, with recovery of 10-88% from feces, indicating that they may be resistant to degradation by gut bacteria also [52].

However, certain studies have shown the presence of intact flavone-mono-C-glucosides in plasma and urine of humans [97], flavone-C-multiglycosides (e.g. vitexin 4"-O-glucoside, vitexin 2"-O-rhamnoside) have been studied to be absorbed intact through the small intestine and distributed to various tissues [6]. Thus, the mechanisms of absorption of different C-linked flavonoids glycosides needs further investigation.

2.8.2. Factors limiting the bioavailability of flavonoids

There are two major factors limiting absorption of flavonoids in the small intestine, thus affecting their bioavailability: 1. metabolism by phase 2 enzymes such as uridine-5'-diphosphate glucuronosyltransferases (UGTs), sulfotransferases (SULTs) and catechol-O-methyl transferases (COMT), and 2. ATP binding cassette (ABC) transporter proteins (breast cancer resistance protein (BCRP/ ABCG2), multidrug resistance protein (MRPs/ABCCs) and P-glycoprotein (*P*-gp/MDR1/ABCB1)) [16-19].

Metabolism of flavonoids by phase 2 enzymes, is part of the xenobiotic detoxification process leading to their excretion via bile or urine by increasing their solubility and molecular weight e.g. glucuronidation, increases the molecular weight and facilitates excretion in bile [98]. Several studies have shown that flavonoids are potent substrates of phase 2 enzymes, with glucuronides, sulfates and methylated forms being the major flavonoid metabolites in circulation [17]. As an example, the flavonol quercetin-4'-O-glucoside was found to be efficiently deglycosylated and rapidly (within 1 h) and extensively metabolized to 18 different conjugates of glucuronides, sulfates or O-methyl derivatives, with 93.6% of ingested dose accumulated in the gastrointestinal tract and only 2.8% present in the plasma, available for absorption by various tissues [99]. Similarly, a study by Hahn et al. 2011, reported relatively poor overall bioavailability of flavone-C-glycosides (only 0.26% of the total amount ingested) in humans fed rooibos tea extracts high in aspalathin (a dihydrochalcone C-glucoside), followed by luteolin-C-glycosides (iso-orientin and orientin) and apigenin-C-glycosides (vitexin and isovitexin) amongst others. Interestingly, though in very low concentrations (0.02-0.4 nmol), intact forms of

isoorientin, orientin, vitexin, and aspalathin were detected in plasma (t_{max} ranging from 1.5 – 3 h), while the urine samples mainly consisted of phase 2 metabolites (glucuronides and O-methyl forms) of aspalathin [97].

ABC transporter proteins are energy dependent efflux pumps (utilizing energy from adenosine triphosphate (ATP)) located in the apical and basolateral membrane of intestinal cells. The transporters located in the apical membrane (P-gp, BCRP and MRP-2) are responsible for effluxing their substrates (both flavonoids and their metabolites)[17] back into the lumen for excretion, whereas, those located on the basolateral side (MRP-1 and MRP3) facilitate their substrate absorption into the blood stream [16]. For example, aglycones quercetin, kaempferol, and C-glucosides, orientin & vitexin were found to be substrates of P-glycoprotein, whereas, conjugates (glucuronides, sulphates) of quercetin, apigenin, chrysin, epicatechin, genistein and C-glycosides 2”-O-β-L-galactopyranosylorientin and 2”-O-β-L-galactopyranosylvitexin were found to be substrates of MRP2 and BCRP [17, 94].

Thus, though the dietary intake of flavonoids is high, their overall bioavailability is low owing to the interplay of phase 2 enzymes and apically located ABC transporters [17]. Thus, inhibition of ABC transporters or reduced metabolism of the flavonoids in the small intestine, could lead to their enhanced bioavailability.

2.8.3. Combination of polyphenols for enhanced bioavailability

Apart from being substrates of ABC transporters and phase 2 enzymes, flavonoids and their metabolites could also function as inhibitors of these factors [16-17, 100].

Flavonoids could competitively inhibit the metabolism or efflux of another substrate thus increasing its bioavailability or they could modify the expression of phase 2 enzymes and ABC transporters at both gene and protein expression levels, thus preventing metabolism/efflux of the compounds. For example, co-incubation of hesperetin (a flavanone) with quercetin (1:1) showed increased hesperetin basolateral concentration by approximately 2X compared to the individual treatment [101]. This effect was attributed to the inhibitory effect of quercetin on ABC transporter BCRP, and competitive inhibition of phase 2 metabolism of hesperetin. Combinations of hesperetin with flavones also showed potential improved bioavailability of hesperetin, though to a lesser extent compared to flavonols. However, combination of hesperetin with flavan-3-ol did not show any effect [101].

Several structure-activity relationships governing interaction of flavonoids with phase 2 enzymes and ABC transporter substrate sites could help explain these outcomes. For example, with respect to SULT activity, across several studies, flavones, flavonols, isoflavones, flavanones, and flavonols were found to be inhibitors of several forms of SULT with a 3,4'-dihroxyflavone, 3'-4'-dihydroxy motif (catechol group) and 7-hydroxyflavone seeming to be essential structural features for enhanced inhibition [17].

A study suggested that the planar structure of a flavonoid as well as its hydrophobicity determines inhibitory effects on P-gp [102]. For example, kaempferol showed greater inhibitory potential of P-gp function than quercetin, followed by flavone baicalein, whereas naringenin which lacks the 2,3-double bond in C-ring had no effect. Further, quercetin-3-O-glucoside as well as rutin did not inhibit P-gp, which the authors

hypothesized to be due to steric hinderance of the glycoside moiety [102]. Hydrophobic interactions have been reported to take place between flavonoid B ring and threonine residues of P-gp substrate binding motifs [103]. Minor contribution of hydrogen bonding has also been identified for flavonoid-P-gp interaction, with potential interactions being established between the carbonyl oxygen of flavonoid derivatives with serine and threonine residues [17, 102-103]. Similarly, for MRP inhibition, flavonols showed highest inhibitory potential owing to their planar structure compared to flavones (the lack of 3-OH, twists the B-ring by 20°). Non-planar flavanones and flavonol B-ring pyrogallol group appeared to be an essential structure for inhibition of MRP2 protein [104]. With respect to BCRP, flavones show higher inhibitory potential than flavonols [105-106].

While above studies define the inhibitory potential of ABC transporters by flavonoids by binding to the substrate binding sites of the proteins, they could also bind to the ATP-binding site of these transporters. ABC transporters derive the energy for their function from ATP, which binds to the nucleotide binding domain [16-17]. Flavonoids have been shown to bind to these domains of ABC transporters, thus preventing binding of ATP and functioning of ABC transporters [16-17]. For example, a study showed that quercetin and apigenin bind to vicinal ATP binding sites of P-gp more strongly than flavanones, isoflavones or glycosylated derivatives [107]. Similarly, flavonoids were shown to compete with nucleotide binding domain of MRP1 for ATP binding with affinity in the order of flavonols (quercetin, galangin) > flavones(apigenin) > flavanones(naringenin) > isoflavones(genistein) [108].

From above studies, it can be deduced that, the C2-C3 double bond conjugation, planar structure and 4-oxo group are essential structural features for enhanced interactions of flavonoids with ABC transporters as well as competitive inhibition of phase 2 metabolizing enzymes. Flavonols and flavones in combination could thus likely show complementary inhibitory effects of phase 2 metabolism and ABC transporters, thus improving their bioavailability in combination.

In agreement with this, a recent study in our lab showed that white sorghum-white cowpea extract combinations (abundant in flavone-apigenin and flavonols-mainly quercetin glycosides) as well as pure compounds apigenin-quercetin combinations synergistically downregulated gene and protein expression of ABC membrane transporters Pgp, MRP2 and BCRP, providing first mechanistic evidence of possible enhanced bioavailability of the flavonoids in combination. This could be a reason for the observed strong synergistic anti-inflammatory action of sorghum-cowpea flavonoids [13, 15]. However, how the combination affects their interaction with phase 2 enzymes, their effect on ABC transporter activity and overall absorption are unknown and needs to be investigated.

C-linked glycosyl flavones show different and poor absorption properties compared to aglycone or O-linked forms. However, Liu et. al (2015) showed flavone-C-glycosides to be substrates for small intestine transporter proteins P-gp and MRP2 [94]. Thus, combination of C-linked flavones with pulse flavonols could also help in improving their bioavailability and needs to be further investigated.

3. MECHANISMS OF SYNERGISTIC ANTI-INFLAMMATORY ACTION OF CEREAL-PULSE FLAVONOID COMBINATIONS IN CACO-2 MODEL *

3.1. Introduction

Epidemiological studies have established an association between whole grain and grain legume intake and reduced risk of inflammation linked chronic diseases, including cardiovascular disease (CVD), cancer and diabetes [37, 110]. Polyphenol composition of the whole grain cereals and pulses likely play a role in the observed benefits. For example, whole wheat, but not refined wheat consumption was reported to reduce inflammation in overweight and obese individuals; this effect was partly attributed to increase in circulating whole wheat-derived phenolic compounds in plasma [111]. Sorghum phenolics were recently shown to reduce postprandial hyperglycemia in humans [112], and adiposity and inflammation in mice fed a high fat diet [113]. Similarly, phenolic compounds in pulses have been shown to directly impact the benefits of pulse intake; e.g., bean phenolics were reported to contribute to protective effects of bean consumption against colon mucosa damage and inflammation in healthy mice [114]. Traditionally, many cultures around the world consume cereals and pulses together as primary staples. Due to their widespread consumption, it is of great interest whether whole grain cereal-pulse combination could provide complementary benefits beyond the protein nutrition. Interestingly, the commonly consumed cereals and pulses also contain structurally different classes of phenolics,

* Reprinted with permission from [109] Ravisankar, S.; Agah, S.; Kim, H.; Talcott, S.; Wu, C.; Awika, J., Combined cereal and pulse flavonoids show enhanced bioavailability by downregulating phase II metabolism and ABC membrane transporter function in Caco-2 model. *Food Chemistry* **2018**. *279*, pg.88-97.

especially the flavonoids [53]. Whole grain cereals like wheat, rye, and sorghum, mainly contain 3-deoxyflavonoids like flavones (apigenin and luteolin derivatives) and flavanones (naringenin, eriodictyol derivatives) [53, 115-117] whereas pulses (e.g., cowpea, common dry beans, chickpea) mainly contain C3-substituted flavonoids (see Fig. 1A), most commonly flavonols and flavanols [59-60, 63, 118]. Such structural differences are known to have important effects on the chemical properties and molecular shape (3-D configuration) of the flavonoids [54, 119-120], thus are likely to affect their biological properties and interaction with relevant cellular receptors.

Few studies have evaluated the combined effect of cereal and pulse polyphenols, or other components as commonly consumed in the diet, on health. Evidence indicates that the predominant flavonoids in cereals (flavones) and pulses (flavonols) show uniquely strong synergistic interaction against inflammatory targets [15, 82]. For example, our recent work showed that combining flavonoids extracted from white sorghum (mostly apigenin derivatives) and white cowpea (mainly quercetin glycosides) strongly downregulated LPS-induced inflammatory targets in CCD-18Co colon myofibroblasts, compared to additive effect [15]. The individual treatments and additive effects produced IC50 values that were on average 15 times (8.3–21 times) higher than the 1:1 combined treatments against LPS-induced nuclear factor-kappa B (NF-κB) and downstream cytokines (tumor necrosis factor α (TNF-α); and interleukins (IL-6 and IL-8)) gene and protein expression in the colon myofibroblasts. Using pure compounds (apigenin and quercetin) to model the interactions revealed similar magnitudes of synergy, confirming the flavonoids in the sorghum and cowpea as the active compounds [15]. Such strong synergy among dietary

compound is not commonly reported in literature and suggests that whole grain cereals and pulses could be strategically combined in foods to amplify their ability to contribute to chronic disease prevention. How these compounds interact needs investigating.

In order for ingested dietary flavonoids to produce biological effects, they must be bioavailable. Phase II enzymes such as uridine-5′- diphosphate glucuronosyltransferases (UGTs) and catechol-O-methyl transferases (COMT) are involved in extensive first pass metabolism of flavonoids by transforming them to more hydrophilic forms for excretion [17]. Secondly, increased efflux of flavonoids from intestinal cells back to the lumen for excretion which is mediated by ATP-binding cassette (ABC) transporter proteins (energy dependent efflux pumps lining the small intestine) also limits their bioavailability [101]. Intestinal ABC transporters involved in flavonoid transport include breast cancer resistance protein (BCRP), multidrug resistance protein (MRP) and Pglycoprotein (*P*-gp/MDR1) [101]. Depending on their structure, flavonoids and their metabolites might act as inducers or inhibitors of ABC transporters and phase II metabolizing enzymes [17, 22]. Thus, these two targets are a logical first step to uncovering how the cereal 3-deoxyflavonoids and pulse flavonoids interact to enhance anti-inflammatory response. We hypothesize that, due to their conjugated structure, combined presence of cereal flavones and pulse flavonols enhances their apparent bioavailability and intracellular accumulation. The objective of this research was to use the Caco-2 monolayer cell model to establish the effect of combining flavones and flavonols common to cereals and pulses, respectively, on their apparent absorption; and whether this effect is governed via action on phase II metabolism and ABC transporter function.

3.2. Materials and methods

3.2.1. Chemicals and reagents

Pure flavonoid compounds used in this investigation were as follows: Apigenin (> 99% purity; the dominant flavonoid aglycone in white sorghum, and most cereals) from Indofine Chemical Company (Hillsborough, NJ, USA) was used to model cereal flavones. Quercetin (98% purity; a common flavonol in pulses) from Sigma-Aldrich Chemicals (St. Louis, MO, USA) was used to model pulse flavonols. Naringenin (an apigenin analog without a C2-C3 double bond (Fig. 3A) abundant in some varieties of sorghum) from MP Biomedicals (Solon, OH, USA) was used to determine the role of the C2-C3 double bond on combined flavonoid interactions. Due to the lack of the C2-C3 double bond, naringenin is chiral at C-2 and thus has a substantially different shape configuration relative to apigenin. Dimethyl sulfoxide (DMSO), Hanks balanced salt solution (HBSS), 2-(*N*-morpholino)ethanesulfonic acid (MES), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

3.2.2. Cell culture

Caco-2 colon adenocarcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and incubated at 37 °C and 5% CO2 in growth media, consisting of Eagle's minimal essential medium (EMEM) 20% fetal bovine serum (FBS), 1% non-essential amino acids, 100 units/mL penicillin G, 100 μg/mL streptomycin, 1.25 μg/mL amphotericin B, and 10mM sodium pyruvate (Sigma-Aldrich Co., St. Louis, MO, USA). Caco-2 cells are a good model for the absorptive and defensive properties of the

intestinal mucosa. These cells, as a model of intestinal barrier, are able to differentiate spontaneously in culture forming monolayers of cells, which express morphological and functional characteristics of the mature enterocytes [121].

3.2.3. Effects of flavonoids on Caco-2 trans-epithelial transport and metabolism

Transport experiment was performed as previously described [101, 122] with slight modifications. Briefly, Caco-2 cells (passage number 33–35), were seeded onto 12 well transwell plate permeable polyester membrane inserts, with 0.4 μm pore diameter (Corning Costar Corp, Cambridge, MA, USA) at a density of 250,000 cells/well. The seeded cells were allowed to grow and differentiate for 21 days from the day of seeding. Formation of monolayer and its integrity was tested by measuring the transepithelial electrical resistance (TEER) values using a EndOhm Volt ohmmeter equipped with a STX-2 electrode (EMD Millipore Corporation, Billerica, MA). Prior to the experiment, the cell inserts were rinsed with Dulbecco's phosphate buffer saline (DPBS) (Gibco, Life technologies, Carlsbad, CA, USA) solution and then incubated with 0.5 mL of HBSS containing 10mM MES on apical side (adjusted to pH 6.5) and 1.5 mL of HBSS containing 1M HEPES (adjusted to pH 7.4) on basolateral side for 30 min, with the different pH solutions used to be more representative of an *in vivo* situation (pH of the gut lumen). TEER values were then measured and only wells with values greater than 450 Ω/cm2 were used for the assay. The experiment was started by adding 400 μL of treatment to the apical side and 1.2 mL of HBSS-HEPES buffer to the basolateral side. Treatments involved quercetin, apigenin and naringenin pre-dissolved in DMSO and diluted to appropriate

concentrations in HBSS-MES buffer (25, 50, 75 and 100 μM of individual compounds and 1:1 (50:50 μM), 1:3 (25:75 μM) and 3:1 (75:25 μM) proportion of compound combination). Basolateral samples collected at 30 min, 1 h and 2 h was stabilized with 4 N HCl and stored at −80 °C until further analysis. The TEER value of the inserts were measured at the end of the experiment also, and only wells with TEER values greater than 250 Ω/cm2 were included in the analysis.

The different ratios of combined treatments did not affect the relative membrane permeability of the compounds ($p > 0.05$), therefore effect of combined treatments on metabolism of the compounds was only assessed in 1:1 ratio, based on method by Williamson et al. [123] with slight modifications. Caco-2 cells were seeded directly onto 6 well plates at a density of 1×10^6 cells/well and allowed to differentiate until monolayer was formed for 12–14 days. On the day of experiment, the cells were rinsed with DPBS and the test compounds (quercetin and apigenin (20 μM) individually and in 1:1 combination) were added in 2 mL of cell culture media. Aliquots of samples (at different time points) were immediately acidified with acetic acid (1 M) and stored at −80 °C to analyze extracellular accumulation of aglycones and metabolites. At the end of the experiment, the cells were washed thoroughly with DPS-methanol (1:1 v/v), scraped and cell pellet was sonicated in 1 mL of methanol to analyze intracellular accumulation of compounds.

3.2.4. Quantification of flavonoids from transport and metabolism experiments

The samples from the transport and metabolism experiments were analyzed for flavonoids and their metabolite profile and concentration on a Waters ACQUITY UPLC Triple Quadrupole MS system (Waters Corp., Milford, MA) equipped with a column heater, sample manager, binary solvent manager, photodiode array eλ (PDA) detector and electrospray ionization (ESI) source. Quercetin was monitored at 360 nm, apigenin was monitored at 340 nm and naringenin at 280 nm. Separation procedure on UPLC was based on conditions recently described (Agah et al., 2017) with slight modification in the gradients as follows: 0–2 min 5% B, 2–8 min 20% B, 8–15 min 70% B, 15–20 min 70% B, 20–23 min 5% B and 23–27 min 5% B. Identification of aglycones and metabolites was done in negative mode with selected ion monitoring on mass channels 301, 315, 381, 395, 463, 491, 572, 653, 667, 477, 557, 571 (representing different likely metabolites of quercetin; glucuronidated, sulfonated or methylated) and on mass channels 269, 621, 635, 445, 525, 539, 459, 363, 349, 283 (representing different likely metabolites of apigenin) with a span of 0.2 Da, dwell times of 0.1 s/channel, cone voltage of 30 V and capillary voltage of 3.5 kV. The gas flow conditions for nitrogen were 800 and 50 L/h for desolvation and at the cone, respectively. Source block temperature and desolvation temperature were set at 150 °C and 400 °C, respectively. Quantification was done by comparing peak area with that of standard curves of authentic standards and molar extinction coefficients for the respective metabolites. The results from transport experiment were reported as apparent permeability coefficients (Papp), calculated as follows:

Papp = (V/ACo) (dC/dt) = cm/s

where V=volume of solution in receiver compartment, A=membrane surface area, Co=initial concentration in donor compartment and dC/dt=change in concentration in receiver solution over time (Walgren, Walle, & Walle, 1998).

3.2.5. Effect of flavonoids on P-gp (MDR1) ATPase activity

Functioning of an ABC efflux transporter depends on the conversion of ATP to ADP by ATPase in order to supply energy to efflux xenobiotics. Thus, we evaluated the modulatory effects of flavonoid combinations on ATPase activity of MDR1 in order to assess if the flavonoid combinations could directly interfere with ABC transporter function. Briefly, ATPase activity of wild type human MDR1 with membranes isolated from transporter expressing *Spodoptera frugiperda* (Sf9) ovarian cells was measured according to manufacturer's protocol (SOLVO biotechnology, Budaors, Hungary) by colorimetric detection of inorganic phosphate, the byproduct of ABC transporter function, as described by Dreiseitel et al. (2009) [124]. Briefly, membranes (4 μg/mL) were pre-incubated at 37 °C with the compounds (Quercetin and apigenin at 10 μM, 1 μM, 1 nM and 1 pM, individually and in 1:1 combination) or the solvent DMSO in the absence and presence of sodium orthovanadate (1.2 mM) which is a known ABC transporter inhibitor, to help distinguish between background ATPase activity and transporter related ATPase activity. Reaction was started by adding MgATP and incubating samples for 10 min at 37 °C. Reactions were stopped by adding color developing agent followed by a blocking reagent. After incubation for 30 min at 37 °C, the absorbance was measured at 600 nm.

Phosphate standards (0.4 and 0.8 nmol Pi per well) were included for optical density (OD) calibration and for calculation of relative ATPase activity, baseline ATPase activity (ctrl 1), vanadate insensitive ATPase activity (ctrl 2), ATPase activity of fully activated membrane (ctrl 3) and vanadate-insensitive ATPase activity of fully activated membrane (ctrl 4). Activation of MDR1 membrane was done using verapamil (40 μM). For calculation of relative activation and inhibition, vanadate-sensitive baseline ATPase activity and maximal vanadate-sensitive ATPase activity after stimulation with verapamil were defined as 0% and 100% transporter ATPase activity, respectively. The following equations were used:

$$\text{Relative activation \%} = \left\{\frac{[\text{Flavonoid} - (\text{Flavonoid} + \text{Vanadate})] - (\text{Ctrl1} - \text{Ctrl2})}{[(\text{Ctrl3} - \text{Ctrl4}) - (\text{Ctrl1} - \text{Ctrl2})]}\right\} \times 100$$

$$\text{Relative inhibition \%} = \left\{\frac{[(\text{Flavonoid} + \text{Reference activator}) - (\text{Flavonoid} + \text{Reference activator} + \text{Vanadate})] - (\text{Ctrl1} - \text{Ctrl2})}{[(\text{Ctrl3} - \text{Ctrl4}) - (\text{Ctrl1} - \text{Ctrl2})]}\right\} \times 100$$

where Flav=flavonoid compound, Ref=reference activator.

3.2.6. Statistical analysis

Experiments were repeated three times. JMP pro 12 or 2009 SAS (Version 9.3, SAS Inst. Inc., Cary, N.C., U.S.A.) were used with one-way Analysis of Variance (ANOVA). Post

Hoc test (Fisher's LSD and Tukey- Kramer HSD) was used to compare treatments means. Dunnett's procedure was used to compare treatment means to respective controls. Significance levels were defined using $p < 0.05$.

3.3. Results and discussion

3.3.1. Effects of combined flavonoids on their membrane permeability

The effective apparent permeability (Papp) of quercetin, apigenin and naringenin, independently and in different combinations (1:1, 1:3 and 3:1) were measured to evaluate the interactive effect on their absorption properties. Controls run without cells indicated that the compounds remained stable in the transport medium over the time period evaluated (Fig 2A). After 2 h, the permeability (50 μM) of quercetin was $1.9 \pm 0.1 \times 10{-}6$ cm/s, apigenin was $4.18 \pm 0.17 \times 10{-}6$ cm/s and naringenin was $25.4 \pm 5.02 \times 10{-}6$ cm/s. There were no significant differences in Papp among different concentrations (25, 50, 75 and 100 μM) for the compounds (Fig 2B, C, D). In general, permeability for naringenin > apigenin > quercetin were in line with previously reported ranges [93].

No significant differences were observed in relative responses of the different ratios of combined treatments (Fig 3&4); therefore only 1:1 (50:50 μM) combinations are discussed. Among the combined treatments, quercetin: apigenin showed the largest increase in permeability compared to the individual treatments (Fig. 3&4). For example, at 2 h, quercetin permeability increased ($p < 0.05$) 3.3 times and apigenin permeability increased 50% in the quercetin-apigenin combined treatments compared to individual treatments (Fig. 3B). Furthermore, over a 2 h period, the absorption (basal concentration)

of quercetin and apigenin in the combined treatments were significantly higher than the absorption of the compounds in individual treatments (Fig. 3C). On the other hand, combination of quercetin or apigenin with naringenin had no significant effect on permeability or apparent absorption of the compounds (Fig. 3D and E).

The membrane permeability data clearly indicates that the structure of the flavonoids has a major impact on how they interact in combination to influence their membrane transport, and thus likely bioavailability. Quercetin and apigenin uniquely enhanced each other's apparent absorption, whereas naringenin combination with either of the molecules did not (Fig. 3). Because quercetin and apigenin are both C2–C3 conjugated (Fig. 3A), they have relatively similar 3-D conformations (B-ring planar relative to heterocyclic (C) ring), and may thus interact with similar protein binding sites [53]. On the other hand, naringenin, due to lack of the C2–C3 conjugation (Fig. 3A), is chiral with a markedly different shape configuration (Bring twisted relative to A and C rings). Naringenin may thus have different protein binding sites, and its cellular uptake may be controlled via independent mechanisms.

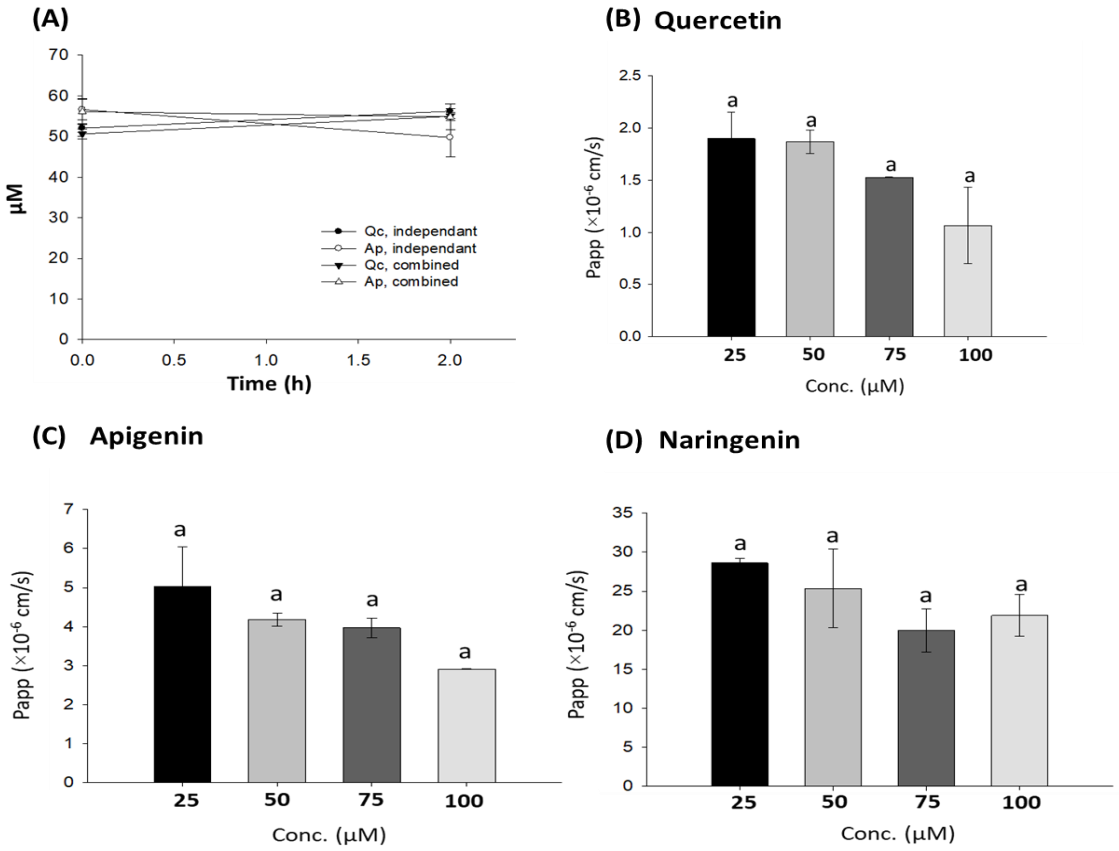


Figure 2: (A) Concentration (µM) of quercetin (Qc) and apigenin (Ap) in independent and combined (1:1) treatments in transport medium without cells over 2 h (B, C, D) Apparent permeability (Papp) of quercetin, apigenin and naringenin at different concentrations (µM) in Caco-2 monolayer model after 2 h of transport. Error bars = ± SD (n=2). Same letter indicates no significant difference between the treatments.

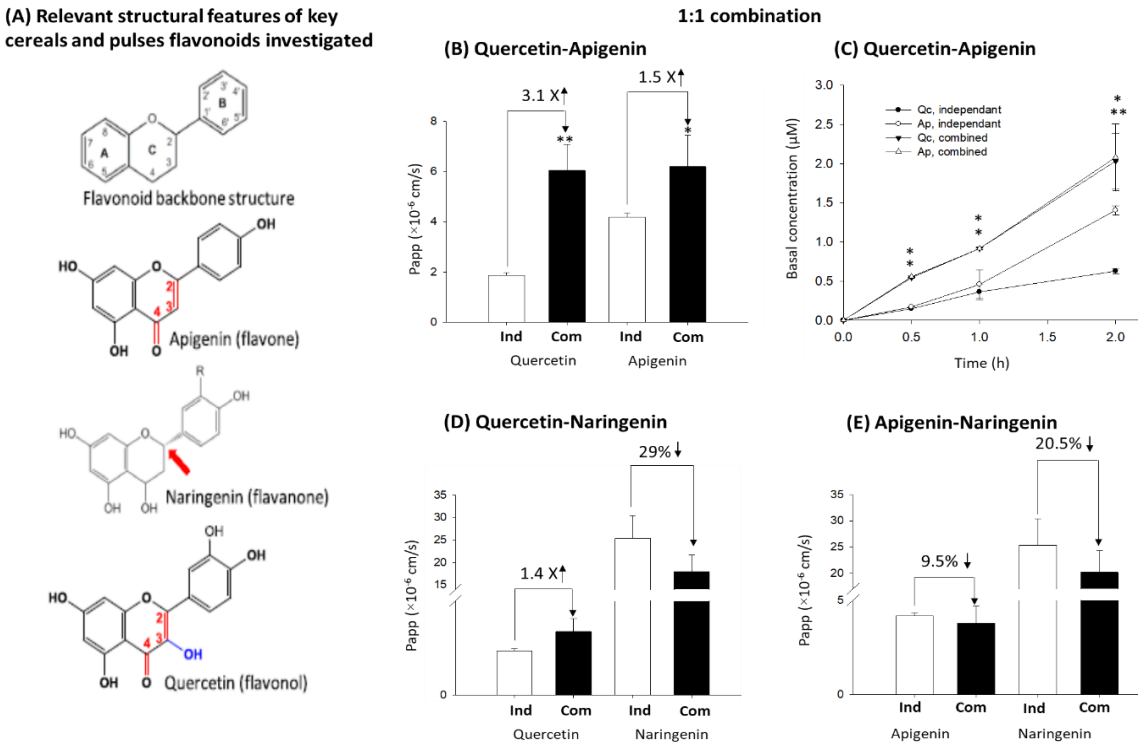


Figure 3: Structural features of key cereal and pulse flavonoids (A) and effect of their combinations (1:1) on apparent permeability (Papp) in Caco-2 monolayer model after 2 h of transport. B, D, E: Ind=independent treatment, Com=combined treatment. (C) Time dependent basal concentration (µM) of quercetin (Qc) and apigenin (Ap) in independent and combined (1:1) treatments. All treatments were at 50 µM concentration. Error bars = ± SD (n=2). *($p < 0.05$), **($p < 0.01$) relative to corresponding independent treatment. Reprinted with permission from [109].

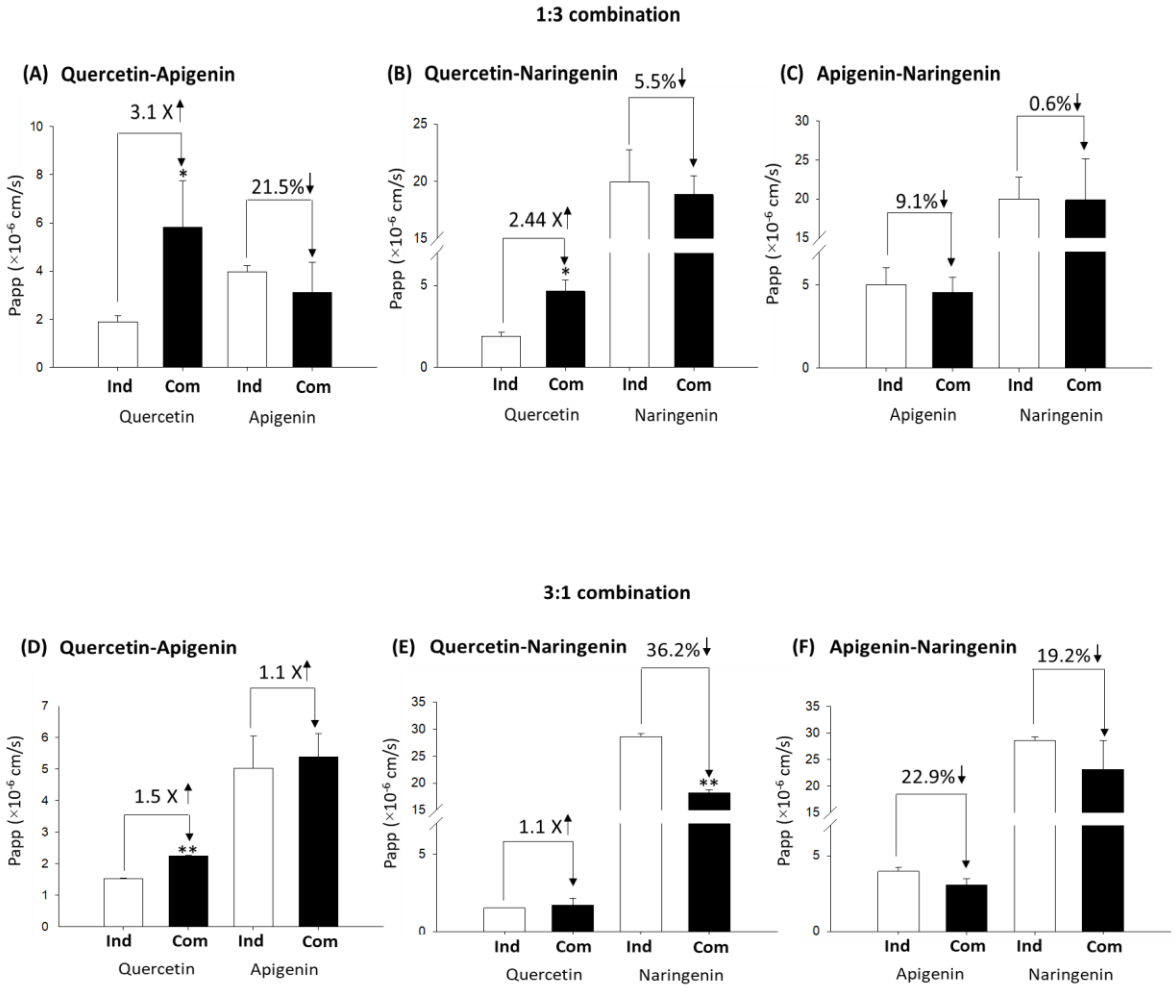


Figure 4: Effect of combinations of key cereal-pulse flavonoids in 1:3 (25:75 µM concentration) proportion (A, B, C) and 3:1 (75:25 µM concentration) proportion (D, E, F) on apparent permeability (Papp) in Caco-2 monolayer model after 2 h of transport. Ind=independent treatment, Com=combined treatment. (Error bars = ± SD (n=2). *($p < 0.05$), **($p < 0.01$) relative to corresponding independent treatment.

3.3.2. Effects of apigenin and quercetin combination on their metabolism

In order to evaluate effect of quercetin-apigenin combination (1:1) on their metabolism, Caco-2 cells were incubated with the compounds for 8 h and extracellular and intracellular accumulation of aglycones (unmodified compound) and metabolites formed was assessed.

As expected, there was a decrease in extracellular concentration of apigenin and quercetin aglycones with time during the incubation period (Fig. 5B and C), suggesting cellular uptake and/or metabolism of the compounds. During the 8 h incubation, the extracellular concentration of the apigenin and quercetin aglycones consistently remained higher in combined treatments compared to individual treatments (Fig. 5B and C). For example, at 8 h, extracellular quercetin aglycone in individual treatment was only 0.47 ± 0.23 μM compared to 2.3 ± 0.9 μM (approx. 5× higher) in combined treatment (Fig. 5B). A similar trend was seen with apigenin. Extracellular apigenin aglycone concentration in individual treatment at 8 h was 6.4 ± 1.22 μM whereas in combined treatment it was 9.14 ± 2.25 μM (Fig. 5C).

Controls run without cells in the culture media indicated both quercetin and apigenin were similarly stable in the media, with no significant difference in their concentration in individual or combined treatments (Fig 5A). Thus, our results suggest that the higher extracellular concentration of apigenin and quercetin in the combined treatments compared to individual treatments is likely due to reduced metabolism of the compounds in combination. This would suggest some interactive inhibition of the phase II metabolizing enzymes. Two major extracellular quercetin metabolites identified were O-methyl-quercetin (*m*/*z* 315) (dominant) and, to a lesser extent, O-methyl-quercetin-glucuronide (*m*/*z* 491). Similar compounds were detected as the major metabolites in plasma of humans after consumption of onions rich in quercetin glycosides [125]. For apigenin, the major metabolite was apigenin-glucuronide (*m*/*z* 445); similarly, Nakamura et al. (2014) reported glucuronides as the major metabolites of isoflavones in humans.

Quantitative analysis of the extracellular metabolites revealed that the concentration O-methyl-quercetin and apigenin-glucuronide increased with time in both individual and combined treatments. However, opposite to what was observed for the unmodified aglycones, combined treatments in general showed lower extracellular accumulation of the metabolites than individual treatments, with both O-methyl-quercetin (1.4 μM vs 2.0 μM) and apigenin-glucuronide (1.6 μM vs 3.0 μM) being significantly lower at 8 h in the combined treatments vs independent treatments, respectively (Fig. 5D and E). This further suggests reduced cellular metabolism of apigenin and quercetin in the combined treatments versus individual treatments.

The other major quercetin metabolite detected, O-methyl-quercetinglucuronide, was only quantifiable at 4–8 h incubation, with maximal accumulation at 4 h (Fig. 5F). In contrast to the other observations, the detectable levels were higher in combined treatments than independent treatments. This suggests a somewhat enhanced secondary metabolism (glucuronidation) of the O-methyl-quercetin metabolite in presence of apigenin. However, the anomaly may be related to inherent reduced oxidative stability of the quercetin glucuronide metabolite in the medium [126], which could suggest a protective role of apigenin against oxidative degradation of the glucuronide. The reduced extent of metabolism of both quercetin and apigenin in combined treatments is likely to lead to increased intracellular accumulation of the parent compounds. This was confirmed by observed higher intracellular content of the both aglycones in combined treatments compared to individual treatments (Fig. 5G). For example, 120 nM quercetin was detected intracellularly in combined treatment, but none was detected in individual treatment.

Likewise, intracellular apigenin accumulation was 40% higher (210 nM) in the combined treatment compared to individual treatment (150 nM) (Fig. 5G). The only metabolite detected intracellularly was O-methyl-quercetin in individual and combined treatments. However, the levels were not significantly different (Fig 5H).

The reduced metabolism and enhanced intracellular accumulation of apigenin and quercetin in the combined treatments (Fig. 5G) could contribute to the enhanced apparent bioavailability observed in quercetin-apigenin combinations (Fig. 3B). The increased apparent bioavailability may partly explain the enhanced anti-inflammatory effects of combined quercetin-apigenin observed in our previous study [15]. A factor that is likely to contribute to the positive interaction of quercetin and apigenin is the difference in their redox reactivity. Due to lack of a C-3 substitution (Fig. 2A), flavones (e.g., apigenin) generally have slower redox kinetics (higher eOH dissociation energy) and thus likely elicit different free radical signaling response versus the C-3 substituted flavonols (e.g., quercetin). The lower eOH dissociation energy of quercetin likely leads to a more rapid phase II signaling response, and may partly explain why it underwent a more rapid phase II metabolism than apigenin (Fig. 5). Nakamura et al. (2014) reported that quercetin was the preferred phase II enzyme metabolite when present together with genistein (the isoflavone analog of apigenin). The rapid metabolism of quercetin also corresponded to a more rapid efflux out of the cell (Fig. 5D and F), thus reducing its apparent bioavailability relative to apigenin (Fig. 3C).

The fact that in presence of apigenin, quercetin absorption increased more than 3× (while apigenin absorption only increased 50%) suggests apigenin readily suppressed factors

(e.g., phase II metabolizing enzymes) responsible for metabolism and/or efflux quercetin. The enhanced uptake of quercetin, may in turn suppress factors responsible for metabolism and/or efflux of apigenin. The metabolite accumulation kinetics (Fig. 5D and E) partly supports this theory. The combined treatment resulted in a rapid reduction in the dominant quercetin metabolite (O-methyl quercetin) accumulation vs individual treatment, whereas reduction in apigenin metabolite accumulation in combined treatment was delayed, and only became apparent after 8 h relative to independent treatment (Fig. 5D and E). The reduced accumulation of Omethyl quercetin in the combined treatment suggests inhibition of catechol-O-methyltransferase (COMT), the enzyme responsible for methylation of quercetin, in presence of apigenin. Lower phase II enzyme expression in Caco-2 was reported for combined quercetin-genistein treatment compared to quercetin alone [125]. However, flavonoids, depending on structure, are known to interact with ABC transporters in ways that can significantly limit or enhance their bioavailability. We thus modeled the interactive effect of these compounds on ABC membrane transporter to gain additional insights.

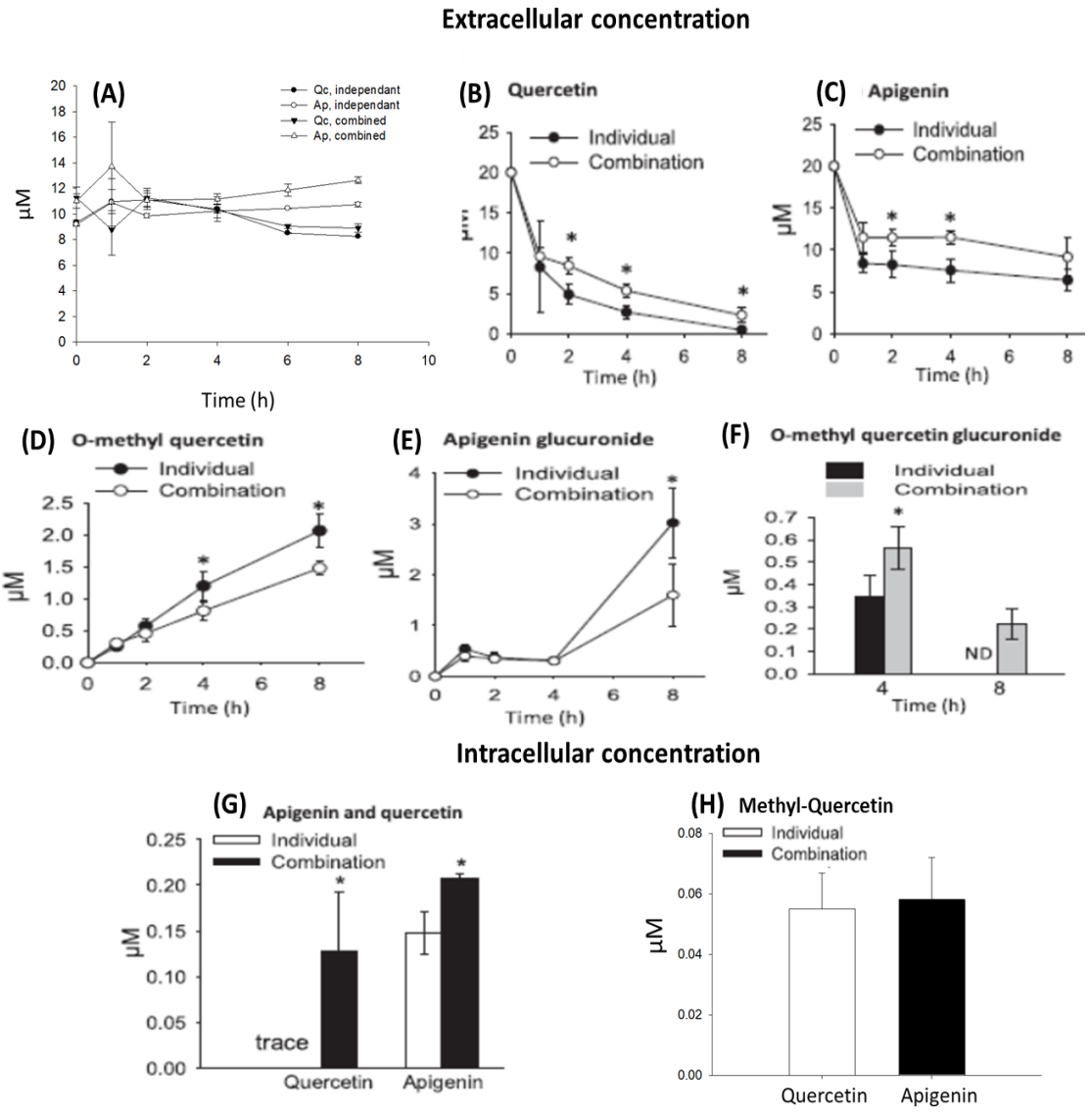


Figure 5: Effect of quercetin and apigenin combinations (20 µM, 1:1) on their metabolism in Caco-2 monolayer model. (A) Compound concentration in cell-culture medium without cells over 8 h (B) and (C): Extracellular accumulation of unmodified aglycones (D–F): Extracellular accumulation of major identified metabolites, G: Intracellular accumulation of unmodified aglycones (H) Intracellular accumulation of quercetin metabolite – methyl-quercetin at 8 h. Error bars = ± SD (n=3). *Significantly different from corresponding individual treatments response ($p < 0.05$). ND, not detected. Parts of figure reprinted with permission from [109].

3.3.3. Effect of apigenin-quercetin combination on Pgp (MDR1) ATPase activity

Previous study from our lab has shown that quercetin-apigenin as well as sorghum-cowpea natural extract combinations could synergistically downregulate ABC membrane transporter expression by 3-40 folds [13]. Besides altering the expression of the ABC transporters, flavonoids can also directly alter the activity of these transporters. This is usually via direct binding to ATP-binding site, thus influencing ABC transporter-specific ATP hydrolysis (ATP conversion to ADP) by ATPase. Activation of ATPase suggests that the compound is actively transported, whereas inhibition indicates direct interference with transporter function [127-128]. To test whether direct effect on activity of ABC transporters was a mechanism that contributed to the apparent enhanced bioavailability of combined apigenin-quercetin treatments, the effect of combining these molecules on P-gp ATPase activity was studied.

The individual and combined treatments at the broad range of concentrations tested (1.0 pM–10 μM), did not increase activity of ATPase beyond baseline, indicating that the compounds did not increase P-gp activity (Fig 6A). However, in the inhibition assay, in the presence of known activator of P-gp, verapamil (40 μM), our results showed a bell-shaped pattern of concentration dependent inhibition of ATPase activity (Fig. 6B). This bell shaped and biphasic concentration-dependent inhibition of P-gp was previously reported, and may be due to the fact that P-gp has multiple binding sites that include transport sites (involved in active transport) and regulatory sites (that modulate P-gp function) [129]. Depending on substrate concentration, these sites are able to switch between high and low affinity conformations [129-130]. Interestingly, at concentrations of ≥1.0 μM,

the individual and combined treatment effects on P-gp activity were similar (Fig. 6B). On the other hand, at lower concentrations (1 nM and 1 pM), combined 1:1 treatments were significantly more effective (by 40.2% and 66.3%, respectively) at inhibiting ATPase activity than individual treatments (Fig. 6B). Our results suggest that at these low concentrations, the inhibition could be non-competitive or positively co-operative thus synergistically decreasing P-gp function.

Our findings suggest that modulating ABC transporter function is an important mechanism by which the flavones and flavonols interact to evade the xenobiotic efflux mechanism and enhance their absorption. The fact that these effects were apparent at relatively low concentrations (picomolar to sub-micromolar range), suggests our data has dietary relevance. For example, based on the levels of the flavones and flavonols in sorghum and cowpea, respectively [14], and assuming a bioavailability of only 5% of the extractable flavonoids, we estimate that combined intake of about 6–12 g of each commodity (9–18 g cooked) is more than adequate to achieve effective concentrations observed in this study.

The interaction of flavonoids with the ABC transporters is complex and can involve multiple mechanisms, including direct binding to ATP binding sites, or via allosteric mechanism. For example, quercetin can act as a substrate, and inhibitor, or inducer of P-gp, MRP2, and BCRP depending on variables like concentration and presence of other xenobiotics [127]. Furthermore, most ABC transporters are known to have multiple binding sites with varying affinities for substrate molecules depending on shape (conformation), hydrophobicity, ionic properties, among others. The consistent effect of both pure

compounds and natural extracts on the ABC transporter function as indicated in a previous study from our lab [15] indicates the predominant flavonoids in whole grain cereals and pulses are potent inhibitors of ABC transporter function in combination, likely via multiple mechanisms.

Both quercetin and apigenin have been shown to bind to vicinal ATP and steroid binding sites of P-gp more strongly than flavanones, isoflavones or their glycosylated derivatives. The binding leads to competition with ATP at the nucleotide binding domains of Pgp, depleting energy required to fuel transport [128]. These studies, however, used concentration of flavonoids (10–50 μM) that are unlikely to be achieved physiologically through normal diet and hence could be misleading. For example, consumption of flavonol containing foods led to 0.3–7.6 μM of flavonols in plasma [131], whereas the maximum apigenin concentration in plasma was reported to reach around 127 nM upon consumption of apiin-rich parsley [132]. Thus, our results are important in demonstrating that physiologically relevant concentrations of these dietary flavonoids can synergistically influence ABC transporter function in significant ways. Furthermore, the ATPase activity data highlights the need to test physiologically relevant concentrations of putative dietary bioactive compounds when assessing their possible mechanisms of action.

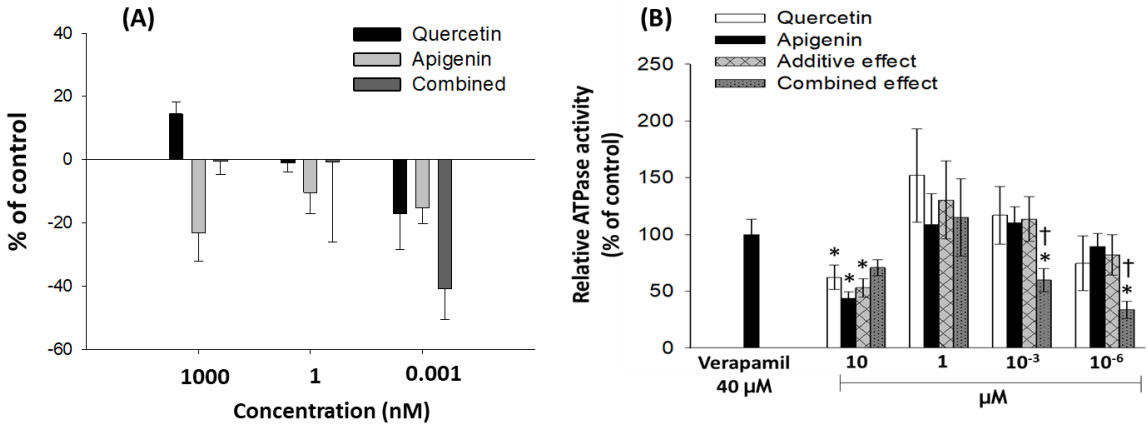


Figure 6: Effect of quercetin and apigenin combinations (1:1) on MDR1 (P-gp) ATPase activity in the absence (A) and presence (B) of verapamil (a known activator of P-gp). Results are expressed as % of activated control. Error bars= ± SD (n=2); *indicates significant difference from verapamil control ($p < 0.05$); †indicates significant difference from respective additive effect at each concentration ($p < 0.05$). Reprinted with permission from [109].

3.4. Conclusion

Results from this study indicate that dietary relevant levels of flavones and flavonols as found in commonly consumed whole grain cereals and pulses, respectively, could have important biological consequences when consumed in combination. These compounds appear to enhance each other's bioavailability primarily through synergistic downregulation of ABC transporter activity by directly binding to ATP-binding site and inhibiting ATPase function. A secondary mechanism is likely via reduced phase II metabolism of the compounds likely due to enhanced or competitive inhibition of phase II metabolizing enzyme activity. The C2-C3 double bond conjugation appears to be an essential feature in the enhanced interactive effects of cereal flavones and pulse flavonols. Thus, the evidence indicates that, depending on their structure, specific dietary flavonoid combinations may provide an effective mechanism to improve flavonoid bioavailability,

and subsequently their beneficial biological effects. Additionally, the synergistic effects observed at low concentrations of the flavonoids suggests that strategic combinations of whole grain cereals and pulses that are naturally low in polyphenols, could provide significantly enhanced health benefits depending on their flavonoid profile. Given whole grain cereals are the only major dietary source of the flavones, our study opens opportunities to explore the relative contribution of these important bioactive compounds on health benefits attributed to whole grain intake.

4. VARIATIONS IN STRUCTURE AND PROFILE OF POLYPHENOLS IN CEREAL GRAINS

4.1. Introduction

Whole grain cereals and pulses are a staple food around the world and a major source of human calorie intake. Several epidemiological studies have shown that consumption of whole grains have been largely associated with positive health benefits such as protection against inflammation, cardiovascular disease, cancer and diabetes [7-8]. Several factors among whole grains contribute to the observed health benefits, among which polyphenols, especially the flavonoid class of compounds are gaining increased attention in recent times [53].

Flavonoids are compounds with a C6-C3-C6 backbone structure and are mainly classified based on their hydroxylation pattern and variations on the heterocyclic ring. Literature shows that commonly consumed whole grain cereals and pulses differ in their flavonoid profile. While cereals such as wheat, barley, sorghum, millets mainly contain 3-deoxy flavonoids, pulses such as common beans, chickpea, cowpea, lentils are mostly abundant in 3-hydroxy flavonoids [53]. Most flavonoids in whole grains are conjugated to glycoside residues (e.g. glucose, galactose, arabinose) either via direct linkage to the aromatic carbon atom (C-linkage) or linked to hydroxyl groups (O-linkage) on flavonoid molecule [46]. Most pulse flavonoids are O-glycoside conjugated (mostly flavonols). Among cereals, sorghum is unique in that it mainly contains, O-linked glycoside or aglycone forms of flavonoids (mainly flavone/flavanones). However, majority of the other cereals, such as wheat, millets, barley contain C-linked glycoside flavonoids (mainly flavones) [53]. This structural variation among different cereals and pulses has a major impact on their beneficial

effects. Recent studies show that combination of cereal and pulse flavonoids have synergistic anti-oxidant and anti-inflammatory activity [15, 44], owing to the differences in the structural features of the flavonoids. Also, as observed in Chapter 3, combination of flavones with flavonols increased their bioavailability as compared to flavone-flavanone or flavonol-flavanone combinations. All this suggests that understanding the structure of compounds in different whole grains is important.

While cereals have been widely profiled for phenolic acids, there is still poor understanding of profile and lack of reliable quantitative data of flavonoids in different cereal grains based on robust methodologies. For example, there is lack of proper identification and quantification of flavonoids in grain rye [56, 133] and major discrepancies exist in the identification of flavonoids in white or brown teff grains [57-58]. Further, there is limited knowledge on differences in profile and content of flavonoids between different wheat varieties (ancient – kamut, spelt, einkorn, farro vs modern wheat) [134] and causes of differences in pigmentation between white and red wheat grains. This gap in literature needs to be improved upon for not only understanding the potential health benefits of the individual grain commodities, but also for their potential interactive effects with pulse flavonoids. This, chapter focusses on establishing the profiles and structural variations among flavonoids in different wheat, sorghum and rye varieties, while Chapter 5 exclusively focusses on profiling different polyphenols in teff grains.

4.2. Materials and methods

4.2.1. Plant materials

Modern wheat (*Triticum aestivum*) grains - Hard white wheat was obtained from Naturals Wheat (Kansas, USA) and red wheat (TAM 231-8) was obtained from Texas A&M AgriLife Research. Ancient wheat grains - einkorn (*Triticum monococcum* var. *monococcum*) was obtained from Jovial (CT, USA), Kamut (*Triticum turanicum*) and Spelt (*Triticum aestivum* var. *spelta*) was obtained from Bob's Red Mill Natural foods (Milwaukie, OR, USA), Farro/Emmer (*Triticum turgidum* var. *dicoccum*) was obtained from Village Harvest (CA, USA). Two sorghum (*sorghum bicolor*) varieties - lemon yellow sorghum (ATX-642) and white sorghum (ATX 635-RTX 436) were grown and harvested in College Station, TX (2016 and 2011, respectively). Rye (*secale cereale*) different varieties belonging to yellow/tan, blue/grey, blue/green and green grain color – Hancock, Spooner, Danko, Rymin, Aroostook, Hazlet, Brasetto, Dacold, KWS Bono, ND Dylan were obtained from North Dakota State University and Musketeer and Prima from University of Minnesota. The whole seeds were ground using UDY mill to pass through a 1 mm mesh sieve and stored at -20 ºC until used.

4.2.2. Chemicals and reagents

All reagents were analytical grade. Apigenin, luteolin, apigenin-7-O-glucoside, luteolin-7-O-glucoside, eriodictyol and its 7-O-glucoside, chrysoeriol were purchased from Extrasynthese (Genay Cedex, France), naringenin was from MP Biomedicals (Solon, Ohio), ferulic acid was from Indofine (Hillsborough, NJ, USA), tricin was from Aobious (MA, USA) and p-coumaric acid, caffeic acid was from Sigma-Aldrich (St. Louis, MO, USA).

4.2.3. Extraction of soluble polyphenols

Extraction of soluble polyphenols was followed according to Liu, Qiu & Beta (2010) [135] and Shumoy & Raes (2016) [57] with slight modifications. Briefly, the ground samples were extracted with 80% methanol (1:5 flour:solvent, w/v) for 2 h with shaking at room temperature. The extracts were centrifuged (10,000 x g for 10 min) using a Heraeus Megafuge 11R Centrifuge (Thermo Fisher Scientific, Asheville, NC) at 20 ºC and the supernatant was transferred to new set of tubes. The residue was further extracted twice (1:2.5 residue:solvent (80% methanol), w/v) each time for 30 min and centrifuged. The supernatants were combined and stored at -80 ºC until further use.

4.2.4. UPLC-Electrospray Ionization (ESI)-MS/MS analysis

Identification and quantification of phenolic acids and flavonoids in the grains was performed according to Ojwang et al. (2012) [60] with slight modifications on a Waters-ACQUITY-UPLC-TQD-MS/MS system (Waters Corp., Milford, MA) equipped with a photodiode array eλ detector and interfaced with a mass spectrometer equipped with a tandem quadrupole (TQD) electrospray ionization (ESI) detector. The separation was performed on a Kinetex C18 column (100 × 2.10 mm, 2.6 µm) (Phenomenex, Torrance, CA) at 40 °C with flow rate of 0.4 mL/min: The mobile phases consisted of 0.05% formic acid in water (solvent A) and 0.05% formic acid in acetonitrile (solvent B) for negative mode ionization and 1% formic acid in water (solvent A) and 1% formic acid in acetonitrile (solvent B) for positive mode ionization. The percentage of solvent B: 0-5 min 5% B, 5-35 min 35% B, 35-38 min 70% B, 38-40 min 70% B, 40-42 min 5% B and 42-45 min 5% B for analysis of polyphenols from rye grains and 0-2 min 5% B, 2-8 min 20% B, 8-15 min 70% B, 15-20 min 70% B, 20-23 min 5% B and 23-27 min 5% B for analysis of polyphenols from

wheat and sorghum. For MS/MS and quantitative analysis, the phenolic extracts were filtered using a syringe filter with a 0.22 µm PTFE membrane and injection volume of 5 µL was used. Flavones were monitored at 340 nm, flavanones at 280 nm, cinnamic acids at 325 nm and anthocyanidins at 520 nm. Mass spectrometric data was acquired in negative mode for all compounds, and in positive mode for certain compounds in rye grains and the microwaved wheat grain extracts. The source, ionization gas flow and data processing conditions was similar to that reported by Ojwang, Dykes, & Awika, 2012[60]. The MS scan was recorded in the range of 100–1000 Da. Parent ion scanning mass parameters was optimized as follows: Capillary voltage was 3 kV; and cone voltage was set at 30 V for negative/positive ionization respectively. The MS/MS scan was optimized as follows: cone voltage of 30 V and collision energy of 15-40 V. Compound identification was done based on matching UPLC retention profile, UV–vis spectra and MS data with authentic standards. Where standards were not available, compounds were identified based on the fragment patterns compared with reports in literature.

Quantification of the compounds was done by interpolating peak areas based on standard curves of corresponding pure compounds. In the case where no standards were available, quantification was done based on the assumption that their molar absorptivity is similar to those of the closest corresponding available compounds similar to the procedure followed by Ojwang, Dykes, & Awika, 2012 [60]. Data is reported on dry basis, based on three separate runs.

4.2.5. Acid catalyzed thermal hydrolysis to test for presence of proanthocyanidins

Based on phenolic profiles obtained from above extraction protocols, we were unable to determine the compounds responsible for the dark reddish-brown pigmentation in red wheat

grains. In the first kind of its study, Kohyama et al. (2017), theorized that flavanols and proanthocyanidins are precursors of the reddish-brown pigments in red wheat seeds being converted to insoluble compounds as the seeds mature [55]. The study was unable to detect the proanthocyanidins in the mature seeds. In order to ascertain if the red wheat mature seeds indeed contained proanthocyanidins in an insoluble form, we used the acid catalyzed thermal hydrolysis and oxidation principle (Porter, Hrstich, & Chan, 1985) [136] to depolymerize and oxidize any present proanthocyanidins to their respective anthocyanidins. Dried residue (from the soluble phenolics extraction) was hydrolyzed using 1% HCl in methanol (1:5 residue:solvent, w/v) using a Microwave Accelerated Reaction System (MARS 5 Xpress, CEM corporation, Matthews, NC). The power was set at 600 W, temperature at 100 °C and reaction was carried out for 10 min. The samples were then centrifuged (10,000 g for 10 min) using a Heraeus Megafuge 11R Centrifuge (Thermo Fisher Scientific, Asheville, NC) at 20 ºC and the supernatant stored at -80 ºC until analysis. Purified proanthocyanidins obtained from sorghum was initially used to develop and validate the hydrolysis method.

4.2.6. Statistical Analysis

Three replications of each treatment were performed. Analysis was done using JMP pro 12 (Version 12.0.1, SAS Institute, Inc., Cary, NC) with one-way Analysis of Variance (ANOVA). Post Hoc test (Fisher's LSD and Tukey-Kramer HSD) was used to compare treatments means. Significance levels were defined using $p < 0.05$.

4.3. Results and discussion

4.3.1. Profiling of polyphenols in different wheat varieties

4.3.1.1. Identification of polyphenols in different wheat varieties

Peak 1, 2, 3 and 4 (t_R 5.911, 6.232, 6.401, 6.692 min, λ_{max} = 272.2, 333.1 and 268.7, 336.6) all had $[M-H]^-$ at m/z 563 with fragment ions at m/z 545 ($[M-H]^-$ - 18 : denoting the loss of a water molecule), 503 (($[M-H]^-$ - 60), 473 ($[M-H]^-$ - 90), 443 ($[M-H]^-$ - 120), 413 ($[M-H]^-$ - 150), 383 (AGly+113) and 353 (AGly+83) which are all typical fragments indicating di-C-linkage of a hexose and a pentose moiety to aglycone apigenin (270 u) (Fig 7 & 8E)) [135]. Since the most common sugar substitutions on di-C-glycoside-flavonoids occurs at position 6 and/or 8 of the aglycone, peaks 1, 2, 3 and 4 were identified as apigenin-6/8-C-pentoside-6/8-C-hexoside isomers (Table 1) and were present in all the different wheat varieties, except einkorn – wherein peak 4 was not detectable (Fig 7). These compounds have been previously identified in charcoal purple wheat [135], durum wheat and kamut [137] and other cereals such as wild rice [138].

Peak 5 and 6 (t_R 7.68 and 7.839 min, λ_{max} = 272.3, 329.5) both had $[M-H]^-$ at m/z 769 with fragment ions at m/z 545 and 425. Fragment ion at m/z 545 indicates the loss of 224 Da from m/z 769, corresponding to a neutral loss of sinapic acid and m/z 425 corresponding to subsequent loss of 120 u from m/z 545, suggesting a C-linked hexoside. Further, similar to interpretation by Liu et al [135], there was absence of a mass loss of 60 Da corresponding to a cross-link cleavage of pentose, thus suggesting that sinapic acid was acylated to the pentose moiety. Thus, peaks 5 and 6 were identified as apigenin-6/8-C-sinapoylpentoside-8/6-C-hexoside isomers (Table 1) similar to that identified by Liu et al in charcoal purple wheat [135]. These compounds were present in all the different wheat varieties (Fig 7).

Thus, the soluble phenolics in the different wheat varieties studied were mainly flavones – primarily apigenin-di-C-glycosides and their acyl (sinapic acid) derivatives. Interestingly, there were no major differences in profile of the flavonoids among the different varieties. These flavones are being identified for the first time in spelt, farro and einkorn wheat grains.

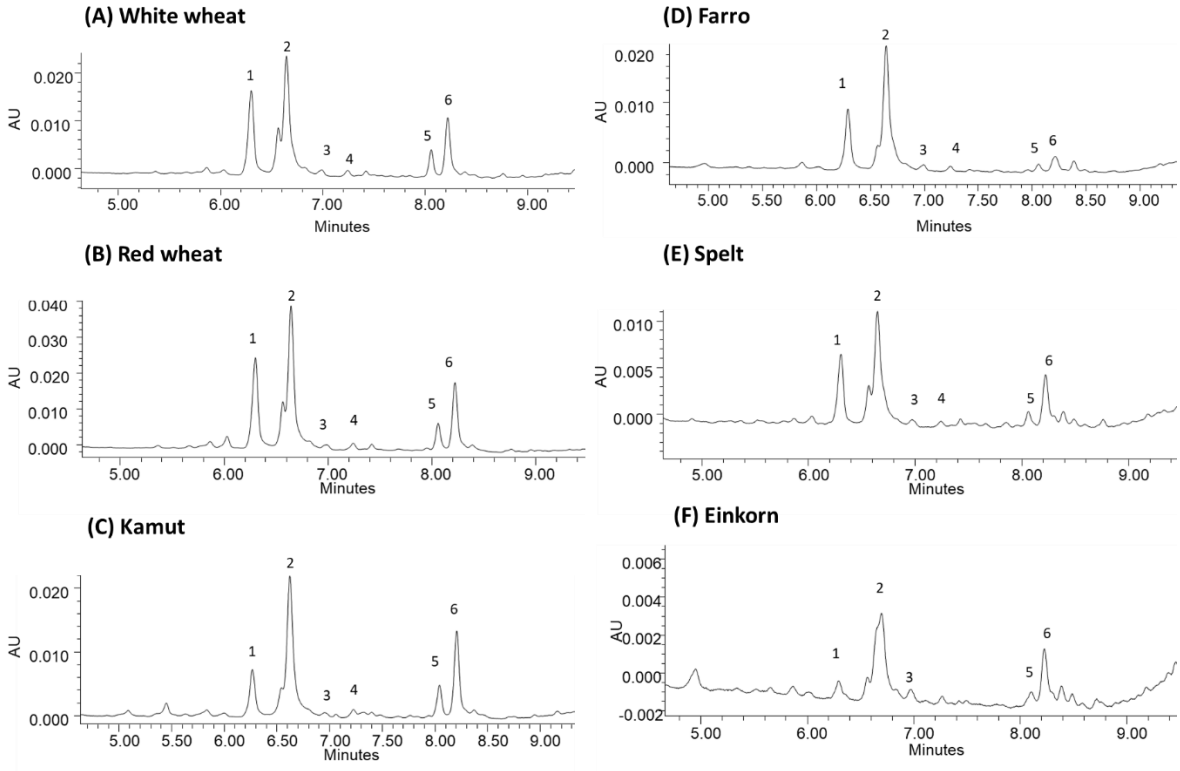
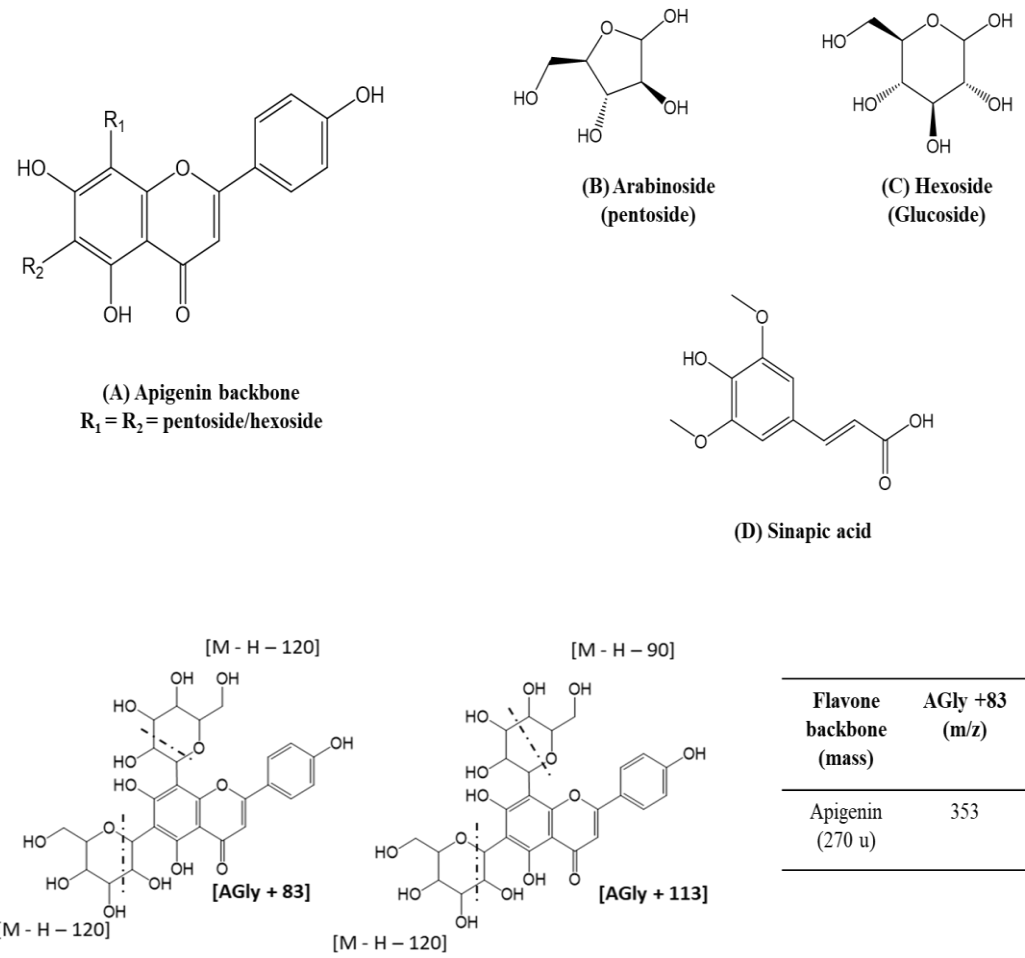


Figure 7: Reverse phase UPLC chromatograms of different wheat varieties monitored at 340 nm. Peak identities are listed in Table 1.



Flavone backbone (mass)	AGly +83 (m/z)	AGly +113 (m/z)
Apigenin (270 u)	353	383

Figure 8: Chemical structures of apigenin and its derivatives identified in different wheat varieties

Table 1: Flavonoids identified in soluble extract of different wheat grain varieties.

Peak no.	Peak retention time (tR)	λmax	[M-H]-	MS/MS fragments	Proposed identification
1	5.911	272.3, 331.1	563	413 (11), 383 (14), 353 (36), 311(69)	Apigenin-6-C-arabinoside-8-C-hexoside isomers
2	6.232	268.7, 336.6	563	545(6), 503 (5), 473 (24), 443 (58), 413(15), 383 (85), 353 (100)	Apigenin-6-C-pentoside-8-C-hexoside isomers
3	6.401	272.3, 333.1	563	503 (38), 473 (14), 443 (11), 413 (47), 383 (97), 353 (100)	Apigenin-6-C-pentoside-8-C-hexoside isomers
4	6.692	272.3, 333.1	563	473 (26), 443 (75), 413 (3), 383 (36), 353 (100)	Apigenin-6-C-pentoside-8-C-hexoside isomers
5	7.68	272.3, 329.5	769	545 (82), 425 (100)	Apigenin-6-C-sinapoylpentoside-8-C-hexoside isomers
6	7.839	272.3, 329.5	769	545 (8), 425 (100)	Apigenin-6-C-sinapoylpentoside-8-C-hexoside isomers

Values in parenthesis represent relative signal intensity as % of base peak

4.3.1.2. Content of flavonoids in different wheat varieties

Though the profile of flavones in the different wheat varieties were similar, they were different in the abundance of total flavones. Red wheat was the highest in total soluble flavone content compared to white wheat (1.3 times higher) and the different ancient wheat varieties (2-10 times higher) (Table 2). Interestingly, all the different ancient wheat varieties had lower total flavone content, compared to the modern wheat varieties. However, this could be specific to varieties of wheat used in this study and further research with multiple genotypes grown in different locations is warranted. Among the different ancient grain varieties, Kamut had the highest total flavone content (84 µg/g) whereas einkorn had the least total flavone content (18 µg/g) (Table 2). Among all the wheat varieties, 65-70 % of flavones were apigenin-di-C-glycosides, and the remaining was acyl derivatives of the di-C-glycosides.

Recently, there is a growing trend of ancient grain consumption being considered as nutritionally important and more healthy [139]. Several studies are thus debating if ancient wheat varieties are nutritionally more rich compared to different modern wheat varieties primarily focusing on fiber, phenolic acids, carotenoids and vitamin contents [134, 139]. A literature review by Peter R. Shewry and Sandra Hey [134] suggested that ancient wheat species einkorn, emmer, Khorasan kamut and spelt were similar in composition to modern cultivars of bread and durum wheat in total phenol, ferulic acid and tocopherol content and were lower in dietary fiber content and higher in carotenoid content [134]. Thus, the study overall concluded that the ancient wheat species may generally not be more healthy than modern wheat [134]. In line with this, our study demonstrates for the first time that the ancient wheat varieties were lower in their total soluble flavonoid content compared to modern wheat varieties. Couple of other studies comparing ancient to modern wheat varieties showed that the ancient and modern wheat varieties were similar in their mean values of total phenolic and flavonoid content [137, 140]. However, the number of identified polyphenols was higher in the ancient wheat grains, showing more diversity in the polyphenol profile in ancient wheat varieties. These studies did not specifically quantify the specific phenol classes [137, 140].

Further studies with multiple genotypes of ancient and modern wheat varieties grown in different locations are needed to thoroughly establish the differences in soluble flavonoid content which could have important implications in defining the bioactive properties of ancient vs modern wheat varieties.

Table 2: Concentration (µg/g, dry weight basis) of identified total soluble flavonoids in different wheat varieties [a].

Peak no.	Proposed identification	White wheat	Red wheat	Kamut	Farro	Spelt	Einkorn
Average µg/g of sample, db							
1	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	29.8 ± 1.1	44.0 ± 2.4	9.9 ± 0.9	12.7 ± 0.5	11.0 ± 0.1	0.5 ± 0.2
2	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	52.2 ± 2.8	78.0 ± 4.0	41.9 ± 2.5	37.7 ± 1.1	28.7 ± 0.6	11.6 ± 0.8
3	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	3.6 ± 0.3	1.1 ± 0.2	0.6 ± 0	0.6 ± 0.1	0.3 ± 0.0	0.2 ± 0.2
4	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	3.6 ± 0.2	2.2 ± 0.5	0.9 ± 0	0.1 ± 0.1	ND	ND
5	Apigenin-6-C-sinapoylpentoside-8-C-hexoside[b]	18.5 ± 0.4	14.4 ± 1.7	7.7 ± 1.3	4.5 ± 0.5	2.5 ± 0.3	0.6 ± 0.5
6	Apigenin-8-C-sinapoylpentoside-8-C-hexoside[b]	33.1 ± 4.0	37.9 ± 3.5	23.5 ± 3.4	1.5 ± 0.2	11.9 ± 0.6	5.3 ± 0.7
	Total	**140.8 ± 8.4 A**	**177.6 ± 11.5 A**	**83.8 ± 8.3 C**	**57.2 ± 2.0 D**	**54.4 ± 1.6 D**	**18.1 ± 2.1 E**

[a]Data are based on UPLC quantification of identified peaks (Table 1) and are expressed as mean ± standard deviation (n=3). [b]As apigenin-7-O-glucoside equivalents. Peaks that were not structurally identified are not included. Different capital letters indicate significant difference among the sample means by Tukey's-HSD ($p<0.05$).

4.3.1.3. Evidence of presence of proanthocyanidins in red wheat grains

Acid catalyzed hydrolysis and oxidation in the presence of heat and alcohol, releases anthocyanidins from their respective proanthocyanidins [136]. Based on this, acid catalyzed thermal hydrolysis of wheat residue (residue remaining after extraction of soluble polyphenols) was conducted to detect the presence of unextractable proanthocyanidins. The hydrolysis of red wheat residue produced a characteristic red colored extract, however, white wheat did not produce the red color. UPLC-MS/MS analysis, showed the presence of a single

peak which was characterized as cyanidin (Parent mass $[M+H]^+$ was at m/z 287, with characteristic ions at 139, 143, 165, 175, 213, and 231 similar to cyanidin) (Fig 9). Thus, it was confirmed that mature red wheat contains proanthocyanidins in a non-extractable form as hypothesized by Kohyama et al. [55] and could indeed be the contributor to the color of red wheat. There are no other studies in literature confirming the presence of proanthocyanidins in red wheat. Further studies need to be done in order to quantify the proanthocyanidin content in red wheat grains. The presence of unextractable proanthocyanidins in red wheat could have a significant impact on the nutritional quality of the food especially with respect to colon metabolism and health [141]. Thus, nutritional and health implications of the unextractable proanthocyanidins needs to be investigated.

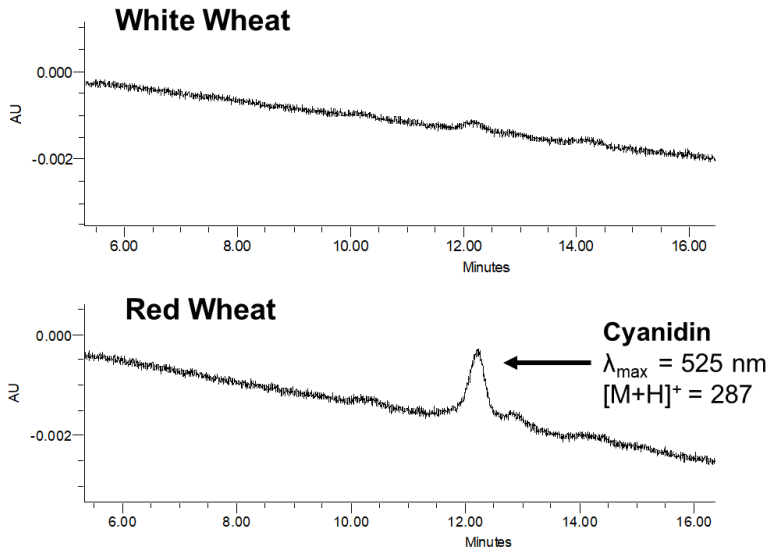


Figure 9: Reverse phase UPLC chromatogram (monitored at 520 nm) of microwaved extract of white and red wheat residues (after extraction of soluble phenolics), demonstrating indirect evidence for presence of procyanidins in red wheat present in a non-extractable form.

4.3.2. Profiling of polyphenols in different sorghum varieties

4.3.2.1. Identification of polyphenols in white and lemon-yellow sorghum

The soluble extracts of white and lemon-yellow sorghum mainly contained phenolic acids in their free and glycerol ester forms mainly as caffeic and ferulic acid derivatives. The flavonoids in white sorghum were mainly dominated by flavone aglycones (apigenin and luteolin) (Fig 10 A). However in lemon-yellow sorghum, the major flavonoids were flavanones (eriodictyol and naringenin) and their O-glucoside forms (Fig 10 B&C).

Peaks 1 and 2 (t_R 4.242 and 4.353 min, λ_{max} = 325.9) had $[M-H]^-$ at m/z 415 (Fig 10). The abundant fragment ions were at m/z 253 (415 – 162 = 253, representing the loss of a O-linked glucoside) and 179, 161 and 135, matching the fragmentation pattern of caffeoylglycerol as reported earlier in white sorghum extracts [142]. Caffeoyl moiety substituted at 2-O-position was found to elute earlier on a reverse phase column than that substituted at 1-O-position [142-143]. Thus, peak 1 was identified as 2-O-caffeoylglycerol-O-glucoside and peak 2 as 1-O-caffeoylglycerol-O-glucoside. Peaks 1 and 2 were identified in white sorghum and peak 2 also in lemon yellow sorghum in this study (Fig 10).

Peaks 3 and 4 (t_R 4.845 and 4.970 min, λ_{max} = 325.9) had $[M-H]^-$ at m/z 253. The UV-visible spectrum and abundant fragment ions were at m/z 161 and 135 (179 – 44 (representing -COOH group) all matching the spectrum and fragmentation pattern of caffeoylglycerol similar to that identified in red and black sorghum previously [25, 117] (Table 3). This compound was identified in white sorghum and lemon-yellow sorghum (Fig 10).

Peak 5 and 6 (t_R 4.99 and 5.09 min, λ_{max} = 294, 315) had $[M-H]^-$ at m/z 468. Major fragment ions included m/z at 332 ($[M-H]^-$ - 136) which represents the loss of a caffeoyl unit with

carboxylic bonds, m/z 306, 161 and 135 which matches fragmentation pattern of N,N'-dicaffeoylspermidine as previously identified in different sorghum extracts [142]. Since both peaks 5 and 6 had same $[M-H]^-$, they were identified as N,N'-dicaffeoylspermidine isomers (Table 3). These compounds were identified in white sorghum (Fig 10).

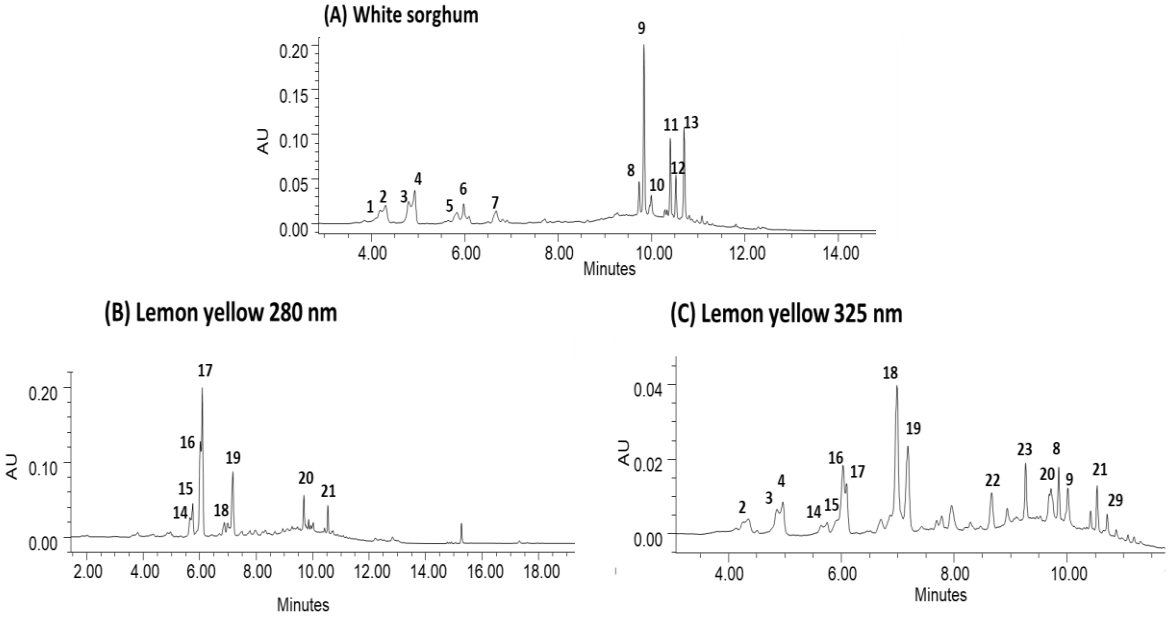


Figure 10: Reverse phase UPLC chromatograms of different (A) White sorghum monitored at 340 nm, (B) Lemon yellow sorghum at 280 nm (C) Lemon yellow sorghum at 325 nm. Peak identities are listed in Table 3 & 4.

Peak 7 (t_R 6.715 min, λ_{max} = 322.3) had $[M-H]^-$ at m/z 193. The major fragment was at m/z 134. Matching retention time, UV-visible spectrum and fragmentation pattern with that of standard, peak 7 was identified as ferulic acid. This compound was identified in a red sorghum variety previously [142] and was identified in white sorghum (Fig. 10).

Peak 8 and 9 (t_R 9.761 and 9.865 min, λ_{max} = 325.9) had $[M-H]^-$ at m/z 415. Major fragment ions included m/z 253 ($[M-H]^-$ - 162 : matching that of caffeoylglycerol), m/z 179 ($[M-H]^-$

- 162 – 74 : matching a caffeoyl fragment), m/z 161 and m/z 135 ($[M-H]^-$ - 162 – 74 – 44 : matching a caffeoyl unit – COOH). Thus, peak 8 and 9 was identified as dicaffeoylglycerol (Table 3) and has been previously identified in sorghum [25, 117]. This compound was identified in white sorghum and lemon-yellow sorghum (Fig 10).

Peak 10 (t_R 10.0.24/21.9 min, λ_{max} = 265, 350) had $[M-H]^-$ at m/z 285 with one dominant fragment ion at m/z 133. This reflects the Retro-Diels-Alder fission of C-ring of luteolin and comparison of the fragmentation pattern with authentic luteolin standard, peak 13 was identified as luteolin (Table 4). This compound has been previously identified in white, black and lemon yellow sorghum [50] and was identified in white sorghum (Fig 10).

Peak 11 (t_R 10.428 min, λ_{max} = 315) had $[M-H]^-$ at 399 with major fragment ions at m/z 253 (caffeoylglycerol), m/z 235, m/z 179 (caffeoyl unit), m/z 163 (coumaroyl unit), m/z 161 and m/z 145. Further, $[M-H]^-$ - 179 – 163 = 57, which corresponds to a glycerol backbone without two substituted -COO units. Thus, peak 10 was identified as p-coumaroyl-caffeoyl-glycerol (Table 3) and have been previously identified in sorghum [25, 117]. These compounds were identified in white sorghum (Fig 10).

Peak 12 (t_R 10.547 min, λ_{max} = 318.8) had $[M-H]^-$ at 429 with major fragment ions at m/z 253 (caffeoylglycerol), m/z 179 (caffeoyl unit), and m/z 134 (fragment of ferulic acid). Since, feruloyl (176) + caffeoyl (162) + glycerol (91) = 429, peak 11 was identified as feruloyl-caffeoyl-glycerol. This peak was identified in white sorghum (Fig 10) in this study and was previously reported in red sorghum [142].

Peak 13 (t_R 10.727/25.10 min, λ_{max} = 265.1, 336.6) had $[M-H]^-$ at m/z 269 with abundant fragment ions at 151, 117 and 119. UV-spectrum, retention time and fragment ions matched that of apigenin standard and hence peak 12 was identified as apigenin (Table 4). This has

been previously identified in white, red and black sorghum [50, 76] . This compound was identified in white sorghum grains (Fig 10).

Peak 14, 15, 16 and 17 (t_R 5.597, 5.704, 5.963 and 6.04 min, λ_{max} = 283) all had $[M-H]^-$ at 449 with a major fragment ion at m/z 287 indicating a loss of 162 amu, corresponding to a O-linked glycoside and further fragment ions at m/z 151 and 135 matching fragmentation pattern of eriodictyol hexoside. Both 5-O and 7-O substituted glycosides of eriodictyol have been reported previously in sorghum [25, 144] and Chang et al 2004 [145] reported that substitution at C-7 increases polarity and results in earlier elution on a reverse phase column. Also, a galactoside elutes before a glucoside on a reverse phase column [60]. Thus peak 14 was identified as eriodictyol-7-O-galactoside, 15 as eriodictyol-5-O-galactoside, 16 as eriodictyol-7-O-glucoside and 17 as eriodictyol-5-O-glucoside (Table 4 and Fig 10) based on a previous study identifying these compounds in lemon yellow sorghum [50].

Peaks 18 and 19 (t_R 6.874 and 7.137 min, λ_{max} = 283) had $[M-H]^-$ at 433 with a major fragment ion at m/z 271 ($[M-H]^-$ - 162 : corresponding to loss of O-linked hexoside) and m/z 151 matching fragmentation pattern of naringenin hexoside. Similar to peaks 16 and 17, since 7-O isomers elutes before 5-O isomer, peak 18 was identified as naringenin-7-O-galactoside and peak 19 as naringenin-5-O-galactoside (Table 4 and Fig 10). These compounds have been previously identified in lemon yellow sorghum [50] and red sorghum [25] and were identified in lemon yellow sorghum in the current study (Fig 10).

Peak 20 and peak 21 (t_R 8.611and 9.243 min, λ_{max} = 375.8 and 357.7) had $[M-H]^-$ at 449 and 433 respectively. Both compounds had dominant ions representing the loss of a hexose unit (162 amu), at m/z 287 (eriodictyol) for peak 20 and m/z 271 (naringenin) for peak 21. Also, both peaks had dominant ions at m/z 151. The UV-visible pattern as well as fragment at m/z

151 suggests that they are chalcone glycosides. Chalcones are precursors for flavanone synthesis in the flavonoid biosynthesis pathway [146-147]. Hence peak 20 was identified as eriodictyol-7-O-galactoside chalcone and peak 21 as naringenin-7-O-galactoside chalcone, based on previous identification in lemon yellow sorghum [142]. These peaks were identified in lemon yellow sorghum in this study (Fig 10).

Peak 22 (t_R 9.648 min, λ_{max} = 286.5) had $[M-H]^-$ at m/z 287 with abundant fragment ions at 151 and 135. UV-spectrum, retention time and fragment ions matched that of eriodictyol standard and hence peak 22 was identified as eriodictyol (Table 4). This has been previously identified in lemon yellow sorghum [76] and red sorghum [25] and was identified in lemon yellow sorghum in the current study (Fig 10).

Peak 23 (t_R 10.659 min, λ_{max} = 290) had $[M-H]^-$ at m/z 271 with abundant fragment ions at 151 and 119. UV-spectrum, retention time and fragment ion matched that of naringenin standard and hence peak 23 was identified as naringenin (Table 4). This has been previously identified in lemon yellow sorghum [76] and red sorghum [25] and was identified in lemon yellow sorghum in the current study (Fig 10).

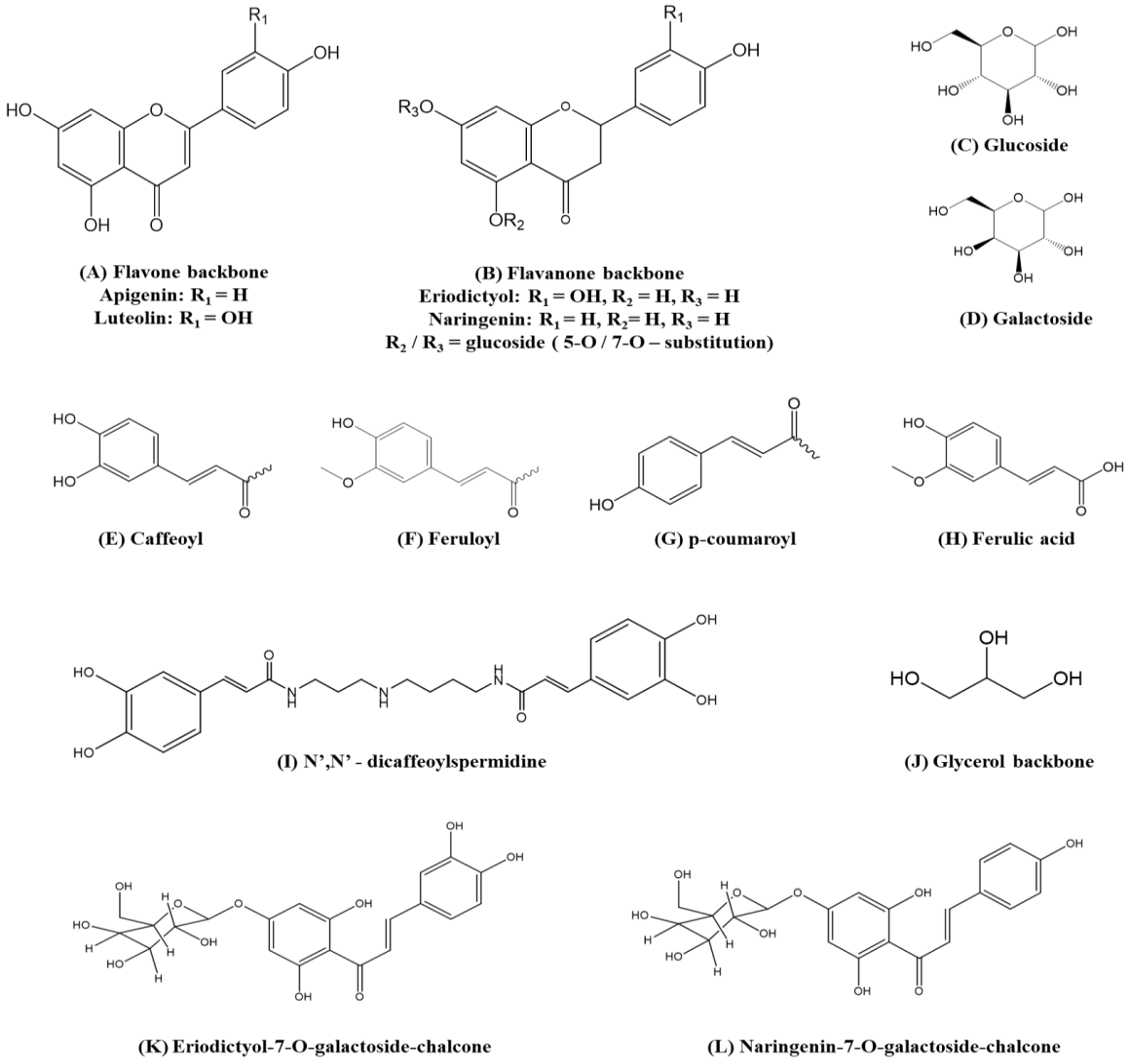


Figure 11: Chemical structures of flavonoids and phenolic acids in different sorghum varieties

4.3.2.2. Quantification of polyphenols in white and lemon-yellow sorghum

The white sorghum extract contained significantly higher levels of phenolic acid derivatives (617 µg/g) compared to lemon yellow sorghum extract (131 µg/g) (Table 3). On the other hand, the total flavonoid content in white sorghum (88 µg/g) was lower than lemon yellow sorghum (1157 µg/g) (Table 4). Furthermore, the flavonoids in white sorghum mainly consisted of aglycone forms of flavones – apigenin and luteolin, while in lemon-yellow sorghum was dominated by aglycone and O-glucoside forms of flavanones – naringenin and eriodictyol. These results were in accordance with that previously studied in the different sorghum grains [142]. The differences in flavonoid profiles could translate to differences in health beneficial outcomes of the grains as evidenced previously wherein white sorghum extract (abundant in flavones) showed higher estrogenic activity compared to red sorghum extract (abundant in flavanone naringenin) [117].

Table 3: Phenolic acids identified in soluble extracts of different sorghum grain varieties and their concentration expressed as μg/g, dry weight basis [a.]

`Peak No.	Peak retention time (tR)	λmax	[M-H]-	MS/MS fragments	Proposed identification	White Sorghum	Lemon Yellow Sorghum
Phenolic acids						**Average μg/g of sample, db**	
1	4.242	322.3	415	179 (10), 161 (100), 135 (34)	2-O-Caffeoylglycerol-O-glucoside[b]	32.3 ± 1.0	ND
2	4.353	322.3	415	253 (47), 179 (13), 161 (56), 135 (25)	1-O-Caffeoylglycerol-O-glucoside[b]	38.2 ± 0.1	17.3 ± 0.5
3	4.845	325.9	253	161 (58), 135 (37)	2-O-Caffeoylglycerol[b]	25.2 ± 1.2	10.1 ± 0.7
4	4.970	325.9	253	161 (58), 135 (37)	1-O-Caffeoylglycerol[b]	31.0 ± 1.7	10.8 ± 0.9
5	5.880	311.6	468	332 (15), 306 (17), 161 (22), 135 (100)	N, N' - Dicaffeoylspermidine[b]	31.1 ± 0.8	ND
6	6.017	318.8	468	332 (22), 306 (32), 161 (31), 135 (100)	N, N' - Dicaffeoylspermidine[b]	29.4 ± 1.9	ND
7	6.715	322.3	193	134 (64)	Ferulic acid[c]	21.2 ± 1.1	ND
8	9.761	325.9	415	253 (6), 179 (58), 161 (20), 135 (100)	Dicaffeoylglycerol[b]	45.5 ± 4.3	36.0 ± 7.5
9	9.865	325.9	415	253 (23), 179 (54), 161 (100), 135 (64)	Dicaffeoylglycerol[b]	137.3 ± 10.4	56.5 ± 10.1
10	10.428	315	399	253 (7), 235 (2), 179 (10), 163 (88), 161 (47), 145(22)	p-coumaroyl-caffeoyl-glycerol[d]	140.6 ±20.9	ND
11	10.547	318.8	429	253 (5), 179 (5), 161 (37), 134 (26)	Feruloyl-caffeoyl-glycerol[c]	85.3 ± 3.0	ND
Total						**617.0 ± 38.3 A**	**130.9 ± 18.2 B**

[a]Data are based on UPLC quantification of identified peaks and are expressed as mean ± standard deviation (n=3). [b]As caffeic acid equivalents; [c] as ferulic acid equivalents; [d] as coumaric acid equivalents. Peaks that were not structurally identified are not included. Values in parenthesis represent relative signal intensity as % of base peak. Different capital letters indicate significant difference among the sample means by Tukey's-HSD ($p<0.05$).

Table 4: Flavonoids identified in soluble extracts of different sorghum grain varieties and their concentration expressed as μg/g, dry weight basis [a.]

Peak no.	Peak retention time (tR)	λmax	[M-H]-	MS/MS fragments	Proposed identification	White Sorghum	Lemon Yellow Sorghum
Flavonoids						**Average μg/g of sample, db**	
12	10.727	265.1, 336.6	269	151 (74), 119 (1), 117 (100)	Apigenin[d]	60.3 ± 10.2	ND
13	10.024	336.6	285	133 (100)	Luteolin[e]	28.0 ± 6.0	ND
14	5.597	283	449	287 (2), 151 (100), 135 (20)	Eriodictyol-7-O-galactoside[b]	ND	68.7 ± 1.4
15	5.704	283	449	287 (3), 151 (100), 135 (47)	Eriodictyol-5-O-galactoside[b]	ND	91.3 ± 0.9
16	5.963	283	449	287 (2), 151 (100), 135 (54)	Eriodictyol-7-O-glucoside[b]	ND	280.9 ± 6.6
17	6.04	283	449	287 (3), 151 (100), 135 (41)	Eriodictyol-5-O-glucoside[b]	ND	327.4 ± 13.8
18	6.874	283	433	271 (28),151 (100)	Naringenin-7-O-galactoside[c]	ND	46.6 ± 1.3
19	7.137	283	433	271 (33), 151 (100)	Naringenin-5-O-galactoside[c]	ND	161.5 ± 5.1
20	8.611	375.8	449	287 (6), 151 (100)	Eriodictyol-7-O-galactoside chalcone[b]	ND	29.6 ± 2.5
21	9.243	357.7	433	271 (26), 151 (100)	Naringenin-7-O-galactoside chalcone[c]	ND	28.9 ± 7.5
22	9.648	286.5	287	151 (10), 135 (100)	Eriodictyol[b]	ND	85.1 ± 12.1
23	10.522	290	271	177 (2), 151 (25), 119 (100), 107 (35)	Naringenin[c]	ND	37.2 ± 4.8
Total						**88.3 ± 16.2 B**	**1157.2 ± 52.1 A**

[a]Data are based on UPLC quantification of identified peaks and are expressed as mean ± standard deviation (n=3). [b]As eriodictyol equivalents; [c] as naringenin equivalents; [d] as apigenin equivalents; [e] as luteolin equivalents. Peaks that were not structurally identified are not included. Values in parenthesis represent relative signal intensity as % of base peak. Different capital letters indicate significant difference among the sample means by Tukey's-HSD ($p<0.05$).

4.3.3. Profiling of polyphenols in different rye varieties

4.3.3.1. Identification of polyphenols in different rye varieties

Profiling of polyphenols in the soluble fraction of different rye grain varieties revealed the presence of 20 major compounds, 19 of which were structurally characterized. Seventeen compounds were flavone derivatives (7 were identified for the first time in rye grains) and 2 compounds were phenolic acid derivatives which were also identified for the first time (Fig 12, Table 5).

Peak 2 (t_R 11.194, λ_{max} = 268.7, 329.5) had $[M-H]^-$ at m/z 593 (Fig 12). The fragments produced had m/z at 503 ($[M-H]^-$ - 90), 473 ($[M-H]^-$ - 120), 383 (aglycone, Agly + 113), 353 (Agly + 83) and 311 (Agly + 41). These are typical fragments for di-C-linked-glycosyl-flavonoids (Agly+83, Agly + 113, Agly+41), indicating the aglycone to be apigenin (270 u) (Fig 13). Further, typical mass losses observed from the cross-link cleavage in hexose moiety ($[M-H]^-$ - 90 and $[M-H]^-$ - 120) indicated the glycosylation with two glucose moieties (Fig 13). The UV-visible spectra was similar to that of apigenin standard. Thus, peak 1 was identified as apigenin-6,8-C-diglucoside (269+162 (glycosyl)+162 = 593), as glucose linkage is the most common linkage identified across cereals and has been previously identified in rye [133].

Peak 3 (t_R 12.119, λ_{max} = 268.7, 346.9) had $[M-H]^-$ at m/z 623 (Fig 12). Fragmentation produced ions at m/z 503 ($[M-H]^-$ - 120), 413 (Agly+113) and 383 (Agly + 83), indicating di-C-glycoside linkage and the aglycone being chrysoeriol (Fig 13). Further, the UV-visible spectra matched that of authentic chrysoeriol standard. Thus, peak 2 was identified as chrysoeriol-6,8-C-diglucoside and has been previously identified in rye [133].

Peaks 4&5 (t_R 13.01 & 13.18, λ_{max} = 268.7, 333.1) both had $[M-H]^-$ at m/z 563 (Fig 12). Fragmentation of the peaks produced ions at m/z 473 ($[M-H]^-$ - 90), m/z 443 ($[M-H]^-$ - 120), 383 (AGly + 113), 353 (AGly + 83), indicating di-C-linkage of a pentose and a hexose moiety to aglycone apigenin (since ($[M-H]^-$ - 60)/ ($[M-H]^-$ - 90) represent cross link cleavage in pentose moiety and ($[M-H]^-$ - 90)/ ($[M-H]^-$ - 120) indicates cross link cleavage in hexose moiety) (Fig 13). Further, the ($[M-H]^-$ - 120) fragment had higher abundance in both peaks, indicating that the hexose moiety is C-6 linked in both peaks as indicated by Ferreres et al. [148]. Since in reverse phase chromatography, a galactose elutes before a glucose, peak 4 was deduced as apigenin-6-C-galactosyl-8-C-arabinoside and peak 5 as apigenin-6-C-glucosyl-8-C-arabinoside and has been previously identified in rye [133].

Peak 6 (t_R 14.225, λ_{max} = 268.7, 329.5) had $[M-H]^-$ at m/z 593 (Fig 12). Fragments produced included m/z 413 (Agly+113), 383 (Agly+83), 353 indicating the aglycone to be chrysoeriol and di-C-linked to a pentose and a hexose moiety (since 299 + 162 (hexosyl) + 132 (pentosy) = 593) (Fig 13). Thus peak 6 was identified as chrysoeriol-6/8-C-glucosyl-6/8-C-arabinoside. This compound has been identified in rye for the first time.

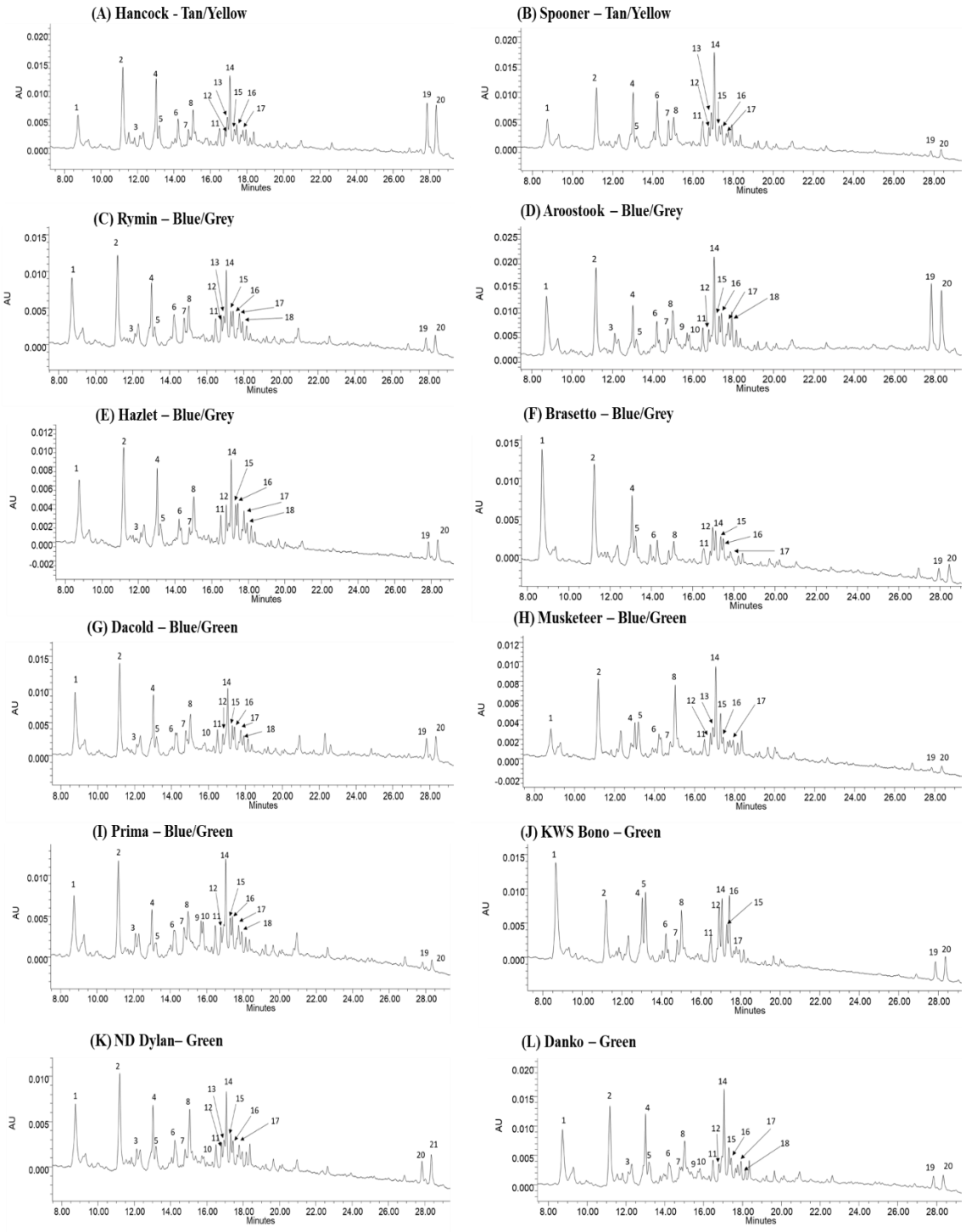
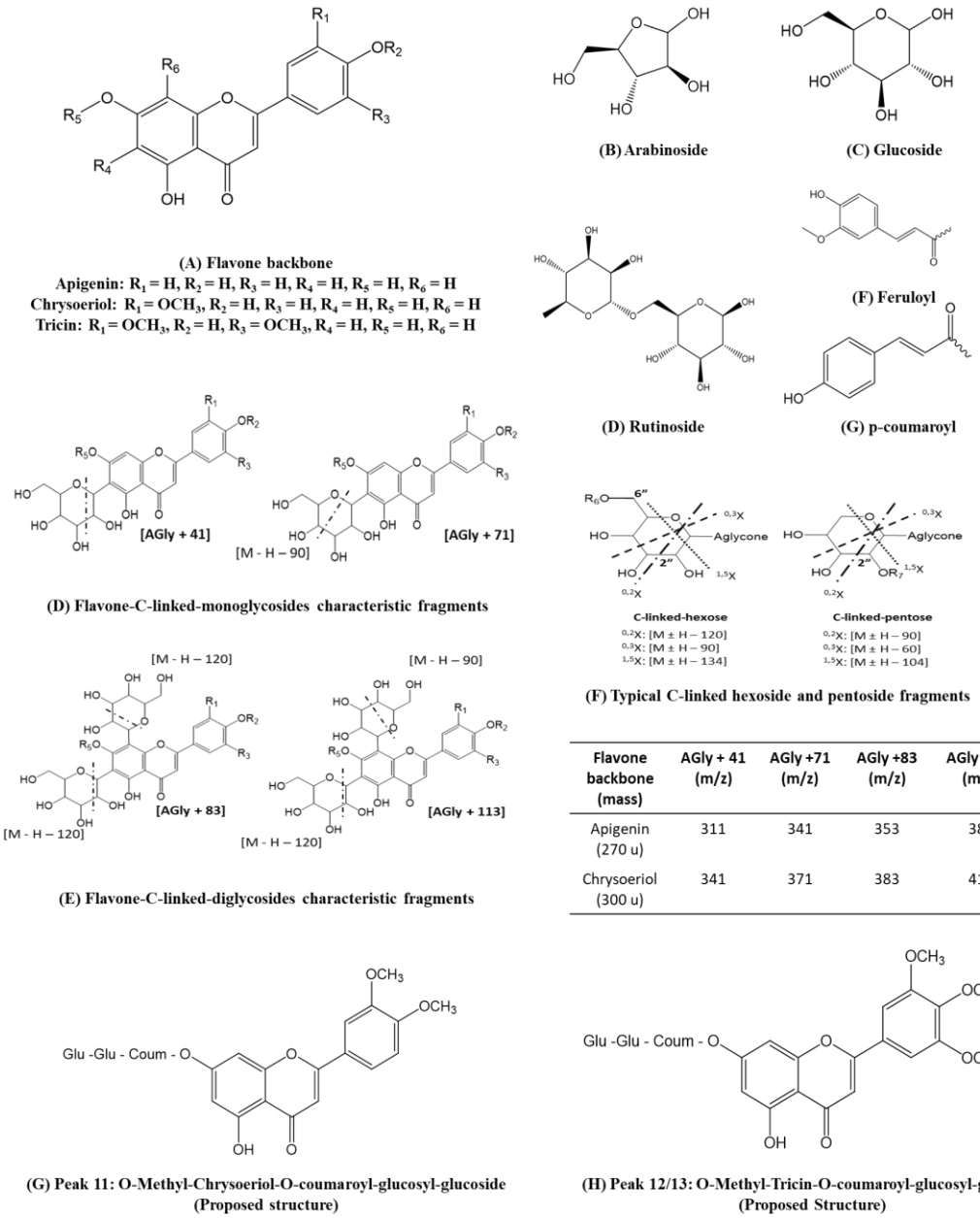


Figure 12: Reverse phase UPLC chromatograms of different rye grain varieties monitored at 340 nm. Peak identities are listed in Table 5.



Flavone backbone (mass)	AGly + 41 (m/z)	AGly +71 (m/z)	AGly +83 (m/z)	AGly +113 (m/z)
Apigenin (270 u)	311	341	353	383
Chrysoeriol (300 u)	341	371	383	413

Figure 13: Chemical structures of flavonoids and phenolic acids in different rye varieties

Table 5: Flavones identified in soluble extract of different rye grain varieties.

Peak no.	Peak retention time (tR)	λmax	[M-H]-/ [M+H]+	MS/MS negative mode fragments	MS/MS positive mode fragments	Proposed identification
1	8.745	315.2	305/307	175 (76), 134 (100)	177 (57), 145 (100)	Unknown - Phenolic acid derivative
2	11.194	268.7, 329.5	593	503 (7), 473 (27), 383 (62), 353 (100), 311 (3)		Apigenin-6,8-C-diglucoside
3	12.119	268.7, 346.9	623	503 (36), 413 (53), 383 (85)		Chrysoeriol-6,8-C-diglucoside
4	13.013	268.7, 333.1	563	473 (3), 443 (3), 383 (47), 353 (77)		Apigenin-6-C-galactosyl-6/8-C-arabinoside
5	13.180	268.7, 333.1	563	473 (4), 443 (16), 383 (85), 353 (100)		Apigenin-6-C-glucosyl-8-C-arabinoside
6*	14.225	268.7, 329.5	593	413 (17), 383 (12), 353 (36), 311 (100)		Chrysoeriol-6/8-C-glucoside-6/8-C-arabinoside
7	14.785	268.7, 329.5	431	341 (16), 311(59), 283 (100)		Apigenin-6/8-C-glucoside
8*	15.001	265.1, 346.9	607	299 (100)		Chrysoeriol-O-rhamnosyl-galactoside
9	15.743	268.7, 346.9	461	371 (24), 341 (100)		Chrysoeriol-6/8-C-glucoside isomer
10	15.901	268.7, 333.1	461	371 (29), 341 (100)		Chrysoeriol-6/8-C-glucoside isomer
11*	16.489	346.9	785/ 787	315 (32), 299 (100)	625 (9), 478 (39), 316 (100), 146 (5)	O-Methyl-Chrysoeriol-O-coumaroyl-glucosyl-glucoside
12*	16.789	346.9	815/816	345 (61), 329 (100)	654 (8), 508 (39), 346 (100), 146 (2)	O-Methyl-Tricin-O-coumaroyl-glucosyl-glucoside isomer

Table 5 continued

Peak no.	Peak retention time (tR)	λmax	[M-H]-/ [M+H]+	MS/MS negative mode fragments	MS/MS positive mode fragments	Proposed identification
13*	16.914	346.9	815	345 (100), 329 (11)	654 (8), 508 (39), 347 (100), 146 (2)	O-Methyl-Tricin-O-coumaroyl-glucosyl-glucoside isomer
14	17.002	346.9	769/ 771	299 (100)	609 (36), 463 (100), 301 (22)	Chrysoeriol-O-rutinoside-glucoside
15	17.302	346.9	799/ 801	329 (100)	639 (52), 493 (100), 331 (17)	Tricin-O-rutinoside-glucoside
16*	17.426	329.5	623/ 625	315 (100), 300 (23)	479 (13), 317 (100), 301 (47), 146 (4)	O-Methyl-Chrysoeriol-O-coumaroyl-glucoside
17*	17.764	346.9	652/ 654	344 (100), 329 (89)	508 (9), 346 (100), 146 (4)	O-Methyl-Tricin-O-coumaroyl-glucoside
18	17.935	340.1	607/ 609	299 (100)	463 (100), 301 (27)	Chrysoeriol-O-rhamnosyl-glucoside
19*	27.730	315.2	413	193 (100), 177 (21), 163 (93), 145 (30), 134 (71), 119 (49)		p-Coumaroyl-feruloyl-glycerol
20*	28.252	322.3	443	193 (54), 175 (25), 149 (21), 134 (100)		di-Feruloyl-glycerol

Values in parenthesis represent relative signal intensity as % of base peak. * indicates compounds being identified for the first time in rye grains

Peak 7 (t_R 14.785, λ_{max} = 268.7, 329.5) had $[M-H]^-$ at 431 (Fig 12). Fragmentation produced ions at m/z 341 (Agly + 71), 311 (Agly + 41) and 283 indicating mono-C-glycosyl linkage to flavonoid and indicating the aglycone to be apigenin (270 u) (Fig 13). Thus, peak 7 was identified as apigenin-6/8-C-glucoside and has been identified in rye before [56].

Peak 8 and Peak 18 (t_R 15.001 & 17.935, λ_{max} = 265.1, 346.9 and 340.1) had $[M-H]^-$ at 607 (Fig 12). In the negative mode, both peaks gave only one characteristic fragment at m/z 299 indicating the aglycone to be chrysoeriol and O-linked to its substituents. In the positive mode, peak 18, provided more information with fragments at m/z 463 ($[M+H]^+$ - 463 = 146, likely indicating a rhamnosyl linkage) and m/z at 301 (463 – 301 = 162, indicating a glucosyl linkage). Thus, peak 8 was tentatively identified as chrysoeriol-O-rhamnosyl-O-galactoside (identified for the first time in rye) whereas peak 18 was then tentatively identified as chrysoeriol-O-rhamnosyl-glucoside (chrysoeriol-O-rutinoside) as galactose elutes before glucose on a reverse phase column [60]. Peak 18 has been previously identified in rye [133].

Peak 9 and peak 10 (t_R 15.743 & 15.901, λ_{max} = 268.7, 346.9 and 268.7,333.1) both had $[M-H]^-$ at 461 (Fig 12). Fragmentation produced ions at m/z 371 (Agly+71) and 341 (Agly+41) which are characteristic mono-C-glycoside linked to flavonoid, indicating the aglycone to be chrysoeriol (300 u) (Fig 13). Thus, peaks 9 and 10 were identified as chrysoeriol-6/8-C-glucoside isomers and has been previously identified in rye [133].

Peaks 11 – 18 were identified based on the fragmentation observed in both positive and negative mode. This helped in distinguishing if the observed mass loss was due to sugar groups or acyl groups (since mass losses of certain sugar and acyl groups overlap with each other – e.g. both rhamnosyl and coumaroyl moieties have a mass loss of 146, glucosyl and caffeoyl groups have a mass loss of 162 and glucuronyl and feruloyl groups have a mass loss

of 176). In the positive mode, there was registration of product ion corresponding to the mass loss of the acid residue, e.g. m/z 146 – corresponding to coumaroyl group, as compared to the negative mode, wherein the acyl group mass loss was not observed [149].

Peak 11 (t_R 16.489, λ_{max} = 346.9) had $[M-H]^-$ /$[M+H]^+$ at m/z 785/787 (Fig 12). Fragmentation in positive mode produced ions at m/z 625 ($[M+H]^+$ - 625 = 162, glucosyl group), 478 (625 – 478 = 147 u), 316 (478- 316 = 162, glucosyl group) and at m/z 146 (indicating the acylation with coumaric acid). In the negative mode the only fragments observed were at m/z 315 and m/z 299, indicating the loss of a methyl group attached to chrysoeriol (300 u). Thus, peak 11 was identified as O-methyl-chrysoeriol-O-coumaroyl-glucosyl-glucoside (Fig 12 & 13G). This compound has been identified in rye for the first time.

Peak 12 & 13 (t_R 16.789 & 16.914 λ_{max} = 346.9) had $[M-H]^-$ /$[M+H]^+$ at m/z 815/816. Fragmentation in positive mode produced fragments at m/z 654 (816 – 654 = 162 u, glucosyl group), m/z 508 (654 – 508 = 146 u), 347 (508 – 346 = 162, glucosyl group) and m/z 146 (indicating the acylation with coumaric acid). In the negative mode the fragments observed were at m/z 345 and m/z 329, indicating the loss of a methyl group attached to tricin (330 u). Thus, peaks 12 and 13 were identified as O-methyl-tricin-O-coumaroyl-glucosyl-glucoside isomers (Fig 12 & 13H). This compound has been identified for the first time in rye.

Peak 14 (t_R 17.002, λ_{max} = 346.9) had $[M-H]^-$ /$[M+H]^+$ at m/z 769/771. Fragmentation in positive mode produced ions at m/z 609 (771 – 609 = 162 u, glucosyl group), 463 (609 – 463 = 146 u, indicating a rhamnosyl group as there was no fragment observed at m/z 146 to indicate it as a coumaroyl moiety), 301 (463 – 301 (chrysoeriol) = 162, glucosyl group).

Thus, peak 14 was identified as Chrysoeriol-O-rhamnosyl-glucosyl-glucoside or chrysoeriol-O-rutinosyl-hexoside. This compound has been previously identified in rye and was a major peak in all the samples (Fig 12) [133].

Peak 15 (t_R 17.302, λ_{max} = 346.9) had $[M-H]^-$ /$[M+H]^+$ at m/z 799/801. Fragmentation in positive mode produced ions at m/z 639 (801 – 639 = 162 u, glucosyl group), 493 (639 – 493 = 146 u, rhamnosyl group, since there was no fragment observed at m/z 146 to indicate it as a coumaroyl moiety), 331 (493 – 331 (tricin) = 162, glucosyl group). Thus, peak 14 was identified as tricin-O-rhamnosyl-glucosyl-glucoside or tricin-O-rutinosyl-hexoside (Fig 12). This compound has been previously identified in rye [133].

Peak 16 (t_R 17.426, λ_{max} = 329.5) had $[M-H]^-$ /$[M+H]^+$ at m/z 623/625. Fragmentation in positive mode produced ions at m/z 479 (625 – 479 = 146 u), 317 (479 – 317 = 162 u, glucosyl group), 301 (317 – 301 = 16, methyl group) and at m/z 146, indicating acyl linkage with coumaric acid. Thus, peak 16 was identified as O-methyl-chrysoeriol-O-coumaroyl-glucoside (Fig 12). This peak has been identified for the first time in rye.

Peak 17 (t_R 17.764, λ_{max} = 346.9) had $[M-H]^-$ /$[M+H]^+$ at m/z 652/654. Fragmentation in positive mode produced ions at m/z 508 (654 – 508 = 146 u), 346 (508 – 346 = 162 u, glucosyl group), and at m/z 146, indicating acyl linkage with coumaric acid. In the negative mode, fragments were observed at m/z 344 and m/z 329 (tricin), indicating the loss of a methyl group attached to tricin. Thus, peak 17 was identified as O-methyl-tricin-O-coumaroyl-glucoside (Fig 12). This peak has been identified for the first time in rye.

Peak 19 (t_R 27.73, λ_{max} = 315.2) had $[M-H]^-$ at m/z 413. Fragmentation produced ions at m/z 163, 145, 119 (163 – 44 (COOH)), indicative of coumaric acid moiety (authentic standard) and 193, 177, 134 (193 – 59, loss of C-COOH group) indicative of ferulic acid moiety. The

remaining mass, 413 – 193 – 163 = 57, which indicated a di-substituted glycerol backbone. Thus, peak 19 was identified as p-coumaroyl-feruloyl-glycerol and has been identified in rye for the first time (Fig 12). Previously it has been identified in sorghum [117].

Peak 20 (t_R 28.252, λ_{max} = 322.3) had $[M-H]^-$ at m/z 443. Fragmentation produced ions at 193, 175, 149 and 134 which are characteristic ferulic acid fragments. Attachment of two ferulic acid moieties to a glycerol backbone, indicates the mass of 443 = 193+193+57 (mass of a di-substituted glycerol backbone). Thus, peak 20 was identified as di-feruloyl-glycerol and has been identified for the first time in rye (Fig 12). Previously it has been identified in sorghum [117].

Thus, 17 flavone derivatives (7 newly identified) and 2 phenolic acid glycerol derivatives (newly identified) were characterized across the different rye varieties. Among the flavone derivatives, majority of them (9) were chrysoeriol derivatives and the remaining were apigenin and tricin derivatives. The apigenin derivatives were mainly present as C-linked glycoside forms, chrysoeriol derivatives were present as O as well as C-linked forms and tricin derivatives were only present as O-linked glycosides. The C-linked forms were present both as mono-C and di-C-linked flavonoid glycosides. This variation in structures among the flavonoids identified in rye (O- and C-linked forms) could affect their bioavailability and hence bioactive properties. In terms of absorption properties, O-linked flavonoids can be cleaved to aglycone form and absorbed in the small intestine, while flavone-C-multiglycosides can be absorbed intact in the small intestine [6]. On the other hand, flavone-mono-C-glycosides are poorly absorbed in the small intestine and mainly reach the colon where they are metabolized to aglycone forms and smaller metabolites [5-6].

Rye is typically consumed as fermented sourdough bread and this process could have differential effects on the flavonoid structures as identified in rye grains which could also influence their bioavailability and potential bioactivity upon consumption. Typically, upon fermentation, O-glycosyl-flavonoids are cleaved to their aglycone form [25] whereas flavone-mono-C-glycosides are not metabolized when fermented [74]. Thus, further studies are required to understand the effect of fermentation on the different flavonoid structures as present in rye grains.

Interestingly, all of the flavonoids were present in all varieties across the different phenotypes, except for the absence of chrysoeriol-O-rutinoside in the yellow/tan phenotypes (Fig 12). Though a majority of the flavonoids were commonly identified across the different rye grains, there were differences in their contents across the phenotypes. This could have important implications in selection and for association of the health benefits with specific rye phenotypes.

4.3.3.2. Content of flavonoids and phenolic acids in different rye varieties

The grain color as well as variety had a major influence on the total flavonoid and phenolic acid content in the different rye grains. Overall, the average total flavone content was highest for Aroostook variety (blue/grey phenotype) (142 µg/g) and the lowest was for Musketeer variety (blue/green phenotype) (57 µg/g) (Table 6&7). Within the phenotypes, there was no variability in total flavone content among the yellow color grain varieties, however, the remaining phenotypes showed significant differences among the varieties. For example, in the blue/grey phenotype, Aroostook had the highest content, followed by Rymin and Hazlet (79 – 86 µg/g) and Brasetto had the lowest (60 µg/g). In the blue green

phenotypes Dacold and Prima had similar contents of flavones (91-104 µg/g), whereas musketeer had almost two times lower content (57 µg/g). Among the green phenotype varieties, KWS Bono and Danko had higher contents of flavones between 122 – 128 µg/g and ND Dylan had the lowest at 90 µg/g. The same trend was observed for phenolic acids also, wherein among all the grains, Aroostook had the highest content (56 µg/g), and Musketeer had the lowest content (3 µg/g) (Table 8).

Among all the phenotypes, the O-linked-glycosyl flavonoids accounted for 50-68% of the total flavone content, with the remaining being the di-C-linked and mono-C-linked flavonoids. Spooner, Aroostook, Dacold, Musketeer, Prima, KWS Bono had higher O-linked flavone contents in the range of 64-68% with the remaining being C-linked flavones. The dominating content of O-glycosyl flavonoids could is relevant as these compounds are typically hydrolyzed into their aglycone forms in the small intestine [17, 51, 85, 87] and show higher absorption potential [92], as compared to the C-linked glycosyl flavonoids, that resist hydrolysis in the small intestine and are absorbed to a lower extent compared to aglycone forms [93-94]. Most of the C-linked glycoside flavonoids reach the colon, where they are deglycosylated or further metabolized into colonic metabolites [5, 96]. However, in general, the absorption potential of compounds from the colon is much reduced compared to the small intestine [95]. Further, rye grains are typically fermented into sourdough products. This could aid the release of the aglycone forms of the flavonoids upon metabolism of the O-glycosides in the process of fermentation [25]. Thus, specific varieties could be appropriately selected based on their content of the different flavonoid forms for delivering enhanced health benefits upon consumption of rye grains.

Table 6: Concentration (μg/g, dry weight basis) of identified flavonoids in soluble extracts of different rye grain varieties of yellow/tan and blue/grey phenotypes [a.]

	Phenotype	**Yellow/Tan**		**Blue/Grey**			
	Variety	**Hancock**	**Spooner**	**Rymin**	**Aroostook**	**Hazlet**	**Brasetto**
Peak no.	**Identification**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**
2	Api-6,8-C-diglc[b]	15.7 ± 1.6	12.6 ± 0.6	12.4 ± 0.8	17.3 ± 0.3	11.3 ± 0.6	15.3 ± 1.5
3	Chry-6,8-C-diglc[c]	4.8 ± 0.3	ND	4.1 ± 0.1	7.8 ± 2.0	2.3 ± 0.4	ND
4	Api-6-C-gal-6/8-C-ara[b]	11.5 ± 1.2	6.0 ± 1.5	3.9 ± 0.3	4.4 ± 0.1	6.3 ± 2.2	8.2 ± 0.6
5	Api-6-C-glu-8-C-ara[b]	1.9 ± .2	0.7 ± 0.1	1.0 ± 0.1	1.4 ± 0.0	1.1 ± 0.1	1.8 ± 0.5
6	Chry-6/8-C-glu-6/8-C-ara[c]	9.4 ± 0.6	20.2 ± 0.7	11.1 ± 0.5	11.9 ± 0.1	7.6 ± 1.2	5.1 ± 0.2
7	Api-6/8-C-glu[b]	ND	2.9 ± 0.2	1.9 ± 0.1	2.7 ± 0.0	0.9 ± 0.1	ND
8	Chry-O-rham-gal[c]	ND	9.7 ± 0.9	10.7 ± 0.9	17.1 ± 2.2	10.9 ± 1.2	6.7 ± 0.1
9	Chry-6/8-C-glu isomer[c]	1.5 ± 0.1	ND	ND	1.4 ± 0.0	ND	ND
10	Chrys-6/8-C-glu isomer[c]	9.8 ± 0.9	ND	ND	1.6 ± 0.1	ND	ND
11	O-Me-Chrys-O-coum-gluc-glu[c]	3.0 ± 0.2	4.7 ± 1.1	1.7 ± 0.5	2.6 ± 0.0	1.1 ± 0.6	0.6 ± 0.0
12	O-Me-Tricin-O-coum-gluc-glu isomer[d]	4.1 ± 0.3	6.0 ± 0.3	4.3 ± 0.0	5.8 ± 0.1	4.0 ± 0.2	3.3 ± 0.2
13	O-Me-Tricin-O-coum-glu-glu isomer[d]	7.3 ± 0.5	12.3 ± 0.5	4.2 ± 0.4	4.9 ± 0.2	ND	ND
14	Chrys-O-rham-glu-glu[c]	25.4 ± 1.5	29.5 ± 0.9	4.2 ± 0.3	30.3 ± 0.4	16.5 ± 0.6	7.1 ± 0.5
15	Tricin-O-rham-glu-glu[d]	7.8 ±0.7	4.7 ± 0.2	11.0 ± 0.6	7.5 ± 0.1	4.4 ± 0.4	4.2 ± 0.2
16	O-Me-Chry-O-coum-gluc[c]	6.9 ± 0.5	4.2 ± 0.2	4.7 ± 0.3	7.8 ± 0.0	4.5 ± 0.7	4.5 ± 0.1
17	O-Me-Tricin-O-coum-gluc[d]	2.8 ± 0.2	2.4 ± 0.1	3.5 ± 0.1	8.9 ± 0.1	5.1 ± 0.2	3.2 ± 0.2
18	Chrys-O-rham-gluc[c]	ND	ND	ND	8.6 ± 0.1	ND	ND
	Total flavonoids	**111.8 ± 8.6 A**	**115.8 ± 3.7 A**	**86.3 ± 1.8 B**	**141.8 ± 1.3 A**	**78.6 ± 4.3 B**	**59.9 ± 3.7 C**

[a]Data are based on UPLC quantification of identified peaks (Table 5) and are expressed as mean ± standard deviation (n=3). [b]As apigenin-7-O-glucoside equivalents; [c]as chrysoeriol equivalents; [d] as tricin equivalents. Peaks that were not structurally identified are not included. Different capital letters indicate significant difference among the sample means by Tukey's-HSD ($p<0.05$).

Table 7: Concentration (μg/g, dry weight basis) of identified flavonoids in soluble extracts of different rye grain varieties of blue/green and green phenotypes [a.]

	Phenotype	**Blue/Green**			**Green**		
Peak	**Variety**	**Dacold**	**Musketeer**	**Prima**	**KWS Bono**	**ND Dylan**	**Danko**
no.	**Identification**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**
2	Api-6,8-C-diglc[b]	10.7 ± .9	7.5 ± 0.7	13.8 ± 1.6	12.2 ± 0.3	12.2 ± 2.8	15.5 ± 0.6
3	Chry-6,8-C-diglc[c]	4.0 ± 0.3	ND	5.0 ± 0.5	ND	5.6 ± 2.5	7.6 ± 2.4
4	Api-6-C-gal-6/8-C-ara[b]	4.2 ± 0.3	3.3 ± 1.6	2.5 ± 0.3	11.7 ± 0.1	4.3 ± 1.3	9.1 ± 3.5
5	Api-6-C-glu-8-C-ara[b]	1.4± 0.1	1.9 ± 0.2	1.2 ± 0.2	7.2 ± 0.1	1.1 ± 0.5	2.6 ± 0.3
6	Chry-6/8-C-glu-6/8-C-ara[c]	8.4 ± 0.2	4.7 ± 0.7	9.5 ± 1.1	7.8 ± 0.2	8.6 ± 1.3	8.9 ± 0.6
7	Api-6/8-C-glu[b]	2.0 ± 0.2	0.5 ± 0.1	1.9 ± 0.1	1.6 ± 0.2	1.3 ± 0.3	1.9 ± 0.1
8	Chry-O-rham-gal[c]	12.3 ± 1.5	12.4 ± 0.8	13.5 ± 2.2	11.2 ± 1.5	ND	19.0 ± 0.9
9	Chry-6/8-C-glu isomer[c]	ND	ND	1.5 ± 1.4	ND	15.0 ± 2.3	0.9 ± 0.1
10	Chrys-6/8-C-glu isomer[c]	0.5 ± 0.8		0.9 ± 0.4		2.7 ± 0.5	2.9 ± 0.7
11	O-Me-Chrys-O-coum-gluc-glu[c]	1.8 ± 0.8	2.5 ± 0.5	2.1 ± 0.7	10.1 ± 0.1	1.6 ± 0.2	7.1 ± 0.6
12	O-Me-Tricin-O-coum-gluc-glu isomer[d]	5.3 ± 0.3	2.7 ± 0.2	5.9 ± 0.7	13.1 ± 0.2	4.0 ± 0.6	4.3 ± 0.3
13	O-Me-Tricin-O-coum-glu-glu isomer[d]	ND	2.5 ± 0.5	ND	ND	3.7 ± 0.3	ND
14	Chrys-O-rham-glu-glu[c]	24.5 ± 3.0	11.7 ± 1.0	6.3 ± 0.6	17.9 ± 0.1	18.4 ± 2.8	32.2 ± 2.7
15	Tricin-O-rham-glu-glu[d]	4.3± 0.5	3.8 ± 0.7	20.7 ± 1.8	20.6 ± 0.0	4.4 ± 0.6	6.8 ± 0.2
16	O-Me-Chry-O-coum-gluc[c]	3.1 ± 2.5	0.9 ± 0.3	5.5 ± 0.6	6.2 ± 0.1	2.8 ± 0.5	3.9 ± 0.8
17	O-Me-Tricin-O-coum-gluc[d]	5.6 ± 0.1	2.0 ± 1.3	4.8 ± 0.6	2.7 ± 0.0	4.2 ± 0.7	2.2 ± 0.2
18	Chrys-O-rham-gluc[c]	3.1 ± 0.1	ND	9.1 ± 0.4	ND	ND	3.3 ± 0.3
	Total flavonoids	**91.2 ± 4.5 A**	**56.5 ± 7.2 B**	**104.1 ± 8.6 A**	**122.2 ± 1.3 A**	**90.0 ± 13.8 B**	**128.1 ± 11.7 A**

[a]Data are based on UPLC quantification of identified peaks (Table 5) and are expressed as mean ± standard deviation (n=3). [b]As apigenin-7-O-glucoside equivalents. [c]as chrysoeriol equivalents. [d] as tricin equivalents. Peaks that were not structurally identified are not included. Different capital letters indicate significant difference among the sample means by Tukey's-HSD ($p<0.05$).

Table 8: Concentration (μg/g, dry weight basis) of identified phenolic acids in soluble extracts of different rye grain varieties of yellow/tan, blue/grey, blue/green, green phenotypes [a.]

	Phenotype	**Yellow/Tan**		**Blue/Grey**			
	Variety	**Hancock**	**Spooner**	**Rymin**	**Aroostook**	**Hazlet**	**Brasetto**
Peak no.	**Identification**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**
19	p-Coumaroyl-feruloyl-glycerol[e]	24.0 ± 3.2	2.4 ± 0.2	6.2 ± 0.3	33.9 ± 5.2	3.3 ± 0.4	3.8 ± 0.5
20	di-Feruloyl-glycerol[f]	18.8 ± 2.2	3.3 ± 0.4	5.6 ± 0.2	22.4 ± 3.0	3.6 ± 0.1	4.4 ± 0.8
	Total phenolic acids	**42.8 ± 5.4**	**5.7 ± 0.6**	**11.8 ± 0.5**	**56.3 ± 8.0**	**6.9 ± 0.5**	**8.2 ± 1.3**
		A	**B**	**B**	**A**	**B**	**B**

	Phenotype	**Blue/Green**			**Green**		
Peak	**Variety**	**Dacold**	**Musketeer**	**Prima**	**KWS Bono**	**ND Dylan**	**Danko**
no.	**Identification**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**	**Avg ± SD**
19	p-Coumaroyl-feruloyl-glycerol[e]	8.4 ± 1.0	1.4 ± 0.2	2.5 ± 0.3	10.3 ± 0.7	9.2 ± 1.4	6.1 ± 0.2
20	di-Feruloyl-glycerol[f]	6.6 ± 0.5	1.4 ± 0.2	2.5 ± 0.3	10.4 ± 0.5	8.7 ± 1.6	4.9 ± 0.1
	Total phenolic acids	**15.0 ± 1.3**	**2.8 ± 0.4**	**5.0 ± 0.6**	**20.7 ± 1.2**	**17.8 ± 3.0**	**11.0 ± 0.2**
		A	**C**	**B**	**A**	**A**	**B**

[a]Data are based on UPLC quantification of identified peaks (Table 5) and are expressed as mean ± standard deviation (n=3). [e] as coumaric acid equivalents. [f] as ferulic acid equivalents. Peaks that were not structurally identified are not included. Different capital letters indicate significant difference among the sample means by Tukey's-HSD ($p<0.05$).

4.4. Conclusions

Results from profiling the different varieties of wheat, sorghum and rye grains suggests that wheat mainly contained flavone-di-C-glycosides. These di-C-glycosides were identified for the first time in spelt, farro and einkorn wheat varieties. Red wheat contained proanthocyanidins in a non-extractable form. The flavonoids in the different sorghum varieties, mainly belonged to the aglycone form of flavones and flavanones or their O-glycoside forms. Rye mainly contained flavone derivatives, both O and C-glycoside linked forms. Among the 19 polyphenols identified across different rye varieites, 9 were identified for the first time in rye grains (7 flavone derivatives and 2 phenolic acid derivatives). Among wheat and rye grains, the profile of flavonoids among the different varieties studied were generally similar. However, there were differences in content of the total flavones with modern red wheat grain and Aroostook (Blue/Grey) rye containing the highest content among the different wheat and rye grains, respectively. Thus, the different cereal grains studied varied in their basic flavonoid structure (flavones, flavanones), with majority belonging to the flavone group of compounds. The profile in cereal grains also varied in terms of glycoside conjugation (O/C-linkage). The differences could could impact the bioavailability and bioactivity of flavonoids from the individual commodities as well as in combination with pulse flavonoids and needs to be further studied.

5. STRUCTURAL PROFILE OF SOLUBLE AND BOUND PHENOLIC COMPOUNDS IN TEFF (*Eragrostis tef)* REVEALS ABUNDANCE OF DISTINCTLY DIFFERENT FLAVONES IN WHITE AND BROWN TEFF VARIETIES [*]

5.1. Introduction

Grain teff (*Eragrostis tef*) is a tropical cereal, native to Ethiopia, where it is a major staple [150]. Generally referred to as tef or teff, this grain is known as the smallest cultivated grain in the world, is adapted to a range of environmental growing conditions and is also less susceptible to attack by pests [151]. This grain is gaining popularity because it is gluten free and very rich in nutrients, especially minerals such as calcium, iron, and magnesium, compared to other grains such as wheat, barley, sorghum and pearl millet [151]. Demand for teff is growing, and it is currently produced in countries such as United States, South Africa, Australia and Canada [152].

Epidemiological evidence has shown that whole grain consumption is strongly associated with reduced risk of chronic diseases such as cancer, diabetes, cardiovascular disease [37-38]. Among the whole grain components that contribute to the benefits are dietary fiber and associated polyphenols. Polyphenols are especially important components of whole grains, because their composition and content have major impact on sensory properties (color, flavor, etc) of whole grain-based products and consumer choices of such

[*] Reprinted with permission from [116] Ravisankar, S.; Abegaz, K.; Awika, J. M., Structural profile of soluble and bound phenolic compounds in teff (*Eragrostis tef*) reveals abundance of distinctly different flavones in white and brown varieties. *Food chemistry* **2018,** *263*, pg. 265-274. Copyright © 2019 Elsevier B.V.

products [153]. For example, in Ethiopia, white teff is preferred over brown teff by most consumers for food [151, 154].

On the other hand, whole grain polyphenols have been shown to directly influence various pathways relevant to chronic disease prevention in important ways, with structural profile of the polyphenols being a major predictor of their biological function [76, 111, 117, 155]. Furthermore, some of the polyphenols, for example, the polymeric proanthocyanidins (condensed tannins), can bind proteins and carbohydrates, significantly reducing their digestibility [67, 156] and thus impacting nutritional quality of food. Therefore, the composition of polyphenols in cereal grains is important, not only from a consumer sensory perspective, but also in helping predict their potential effect on nutrient bioavailability, and chronic disease prevention.

There is surprisingly little that is known about the phenolic profile of teff; and as yet, the compounds responsible for the intense pigmentation in the brown teff pericarp are unknown. The few studies available on teff [57-58, 157-158] are very limited in scope and provide little structural information and/or reliable quantitative data on teff phenolics. Not surprisingly, there is little agreement on the type of compounds identified across the studies. Thus, the phenolic composition of teff remains largely unknown. In this study, we use UPLC-tandem quadrupole MS/MS to profile the structure and content of soluble (free) and bound polyphenols in white and brown teff grains grown in Ethiopia and USA.

5.2. Materials and methods

5.2.1. Plant materials

White and brown teff grains grown in Idaho, USA (Shiloh farms, PA, USA) in 2016 were commercially purchased. In addition, white and brown teff were purchased from a local

market in Hawassa, Ethiopia in 2017. The whole seeds were ground using UDY mill to pass through a 1 mm mesh sieve and stored at -20 ºC until used.

5.2.2. Chemicals and reagents

All reagents were analytical grade. Apigenin, apigenin-7-O-glucoside, luteolin, luteolin-7-O-glucoside and cyanidin chloride were purchased from Extrasynthese (Genay Cedex, France), ferulic acid was from Indofine (Hillsborough, NJ, USA) and p-coumaric acid was from Sigma-Aldrich (St. Louis, MO, USA).

5.2.3. Extraction of soluble and bound polyphenols

Extraction of soluble phenolics was performed according to Liu, Qiu, & Beta (2010) [135] and Shumoy & Raes (2016) [150], with slight modifications. Ground teff was extracted in 80% methanol (1:5 flour:solvent, w/v) for 2 h with shaking at room temperature (Standard Analog Shaker- speed 5, VWR, Radnor, PA). The extracts were centrifuged (10,000 g for 10 min) using a Heraeus Megafuge 11R Centrifuge (Thermo Fisher Scientific, Asheville, NC) at 20 ºC and the supernatant was transferred to new set of tubes. The residue was further extracted twice (1:2.5 residue:solvent (80% methanol), w/v) each time for 30 min and centrifuged. The supernatants were combined and stored at -80 ºC until further use.

Extraction of bound phenolics was performed according to Liu, Qiu, & Beta, (2010)[135] and Shumoy, Gabaza, Vandevelde, & Raes, (2017) [68] with slight modifications. Briefly, 1 g of dried residue (from the soluble phenolics extraction) was hydrolyzed in 30 mL of 2 M NaOH for 30 min at 60 °C. The samples were then centrifuged at 10,000 g for 30 min and the supernatant was decanted into new set of tubes. The pH of the supernatant was adjusted to

1.5-3 by using 6 N HCl and extracted once with 30 mL hexane to remove lipids. The phenolics were then extracted twice with ethyl acetate (once with 30 mL and 2nd time with 20 mL). The ethyl acetate fractions were combined and dried using rotary evaporator under vacuum (Buchmann R110 Rotavapor, Westbury, NY). The residue was dissolved in 1 mL 80% methanol and stored at -80 ºC until further use.

5.2.4. Acid catalyzed thermal hydrolysis to test for presence of proanthocyanidins

Based on phenolic profiles obtained from above extraction protocols, we were unable to determine the compounds responsible for the dark reddish-brown pigmentation in the brown teff pericarp. In other grains like sorghum and finger millet, such pigmentation is typically a function of a pigmented testa containing proanthocyanidins. We thus theorized that insoluble condensed tannin-like compounds were likely responsible. To test this, we used the acid catalyzed thermal hydrolysis and oxidation principle to depolymerize and oxidize any present proanthocyanidins to their respective anthocyanidins [136]. Dried residue (from the soluble phenolics extraction) was hydrolyzed using 1% HCl in methanol (1:5 residue:solvent, w/v) using a Microwave Accelerated Reaction System (MARS 5 Xpress, CEM corporation, Matthews, NC). The power was set at 600 W, temperature at 100 °C and reaction was carried out for 10 min. The samples were then centrifuged (10,000 g for 10 min) using a Heraeus Megafuge 11R Centrifuge (Thermo Fisher Scientific, Asheville, NC) at 20 ºC and the supernatant stored at -80 ºC until analysis. Purified proanthocyanidins obtained from sorghum was initially used to develop and validate the hydrolysis method.

5.2.5. Ultra Performance Liquid Chromatography-Tandem Quadrupole MS/MS analysis

Identification and quantification of polyphenols in teff grains was performed according to Ojwang, Dykes, & Awika (2012) [60] with slight modification on a Waters-ACQUITY-UPLC-TQD-MS/MS system (Waters Corp., Milford, MA) equipped with a photodiode array eλ detector and interfaced with a mass spectrometer equipped with a tandem quadrupole (TQD) electrospray ionization (ESI) detector. The separation was performed on a Kinetex C18 column (100 × 2.10 mm, 2.6 µm) (Phenomenex, Torrance, CA) at 40 °C with flow rate of 0.4 mL/min: The mobile phases consisted of 0.05% formic acid in water (solvent A) and 0.05% formic acid in acetonitrile (solvent B) for phenolic acid and flavone analysis whereas 1% formic acid in water (solvent A) and 1% formic acid in acetonitrile (solvent B) was used for anthocyanin analysis. The percentage of solvent B was 0-5 min 5% B, 5-35 min 35% B, 35-45 min 70% B, 45-50 min 70% B, 50-53 min 5% B and 53-60 min 5% B. For MS/MS analysis, the phenolic extracts were filtered using a syringe filter with a 0.22 µm PTFE membrane and the injection volume was 2 µL, whereas for quantification, the combined supernatants were injected at a volume of 5 µL. Flavones were monitored at 340 nm, other phenolic acids at 280 nm and 325 nm, anthocyanins at 480 nm and 520 nm and mass spectrometric data were acquired in negative mode for phenolic acids and flavones and in positive mode for anthocyanins. The source, ionization gas flow and data processing conditions were similar to that reported by Ojwang, Dykes, & Awika (2012). The MS scan was recorded in the range of 100–1000 Da. Parent ion scanning mass parameters were optimized as follows: Capillary voltage was 3.5/3 kV; and cone voltage was set at 30 V for positive/negative ionization respectively. The MS/MS scan was optimized as follows: cone

voltage of 30 V and collision energy of 15-40 V. Compound identification was done based on matching UPLC retention profile, UV–vis spectra and MS data with authentic standards. Where standards were not available, compounds were identified based on the fragment patterns compared with reports in literature.

Quantification of the compounds was done by interpolating peak areas based on standard curves of corresponding pure compounds. In the cases where no standards were available, quantification was done based on the assumption that their molar absorptivity was similar to those of their monoglucosides (apigenin-7-O-glucoside for apigenin derivatives, luteolin-7-O-glucoside for luteolin derivatives, ferulic acid for the monomers and ferulic acid dehydrodimers and coumaric acid for the corresponding monomer) similar to the procedure followed by Ojwang, Dykes, & Awika (2012). Data was reported on dry basis, based on three separate runs.

5.2.6. Statistical Analysis

Three replications of each treatment were performed. Analysis was done using JMP pro 12 (Version 12.0.1, SAS Institute, Inc., Cary, NC) with one-way Analysis of Variance (ANOVA). Post Hoc test (Fisher's LSD and Tukey-Kramer HSD) was used to compare treatments means. Significance levels were defined using $p < 0.05$.

5.3. Results and discussion

5.3.1. Polyphenols in soluble fraction of white and brown teff

UPLC chromatograms at 280, 325, 340 and 520 nm of polyphenols in soluble fraction of white and brown teff grains revealed the presence of 28 peaks which were

structurally characterized (Table 9). All these 28 compounds are being reported in teff grains for the first time, to the best of our knowledge. They consisted of 26 flavones and 2 phenolic acids. Surprisingly, there was little overlap between the flavone peaks in white and brown teff grains, indicating distinctly different flavonoid biosynthetic pathways (Fig 14). On the other hand, the profile within teff phenotype for grains grown in Ethiopia vs USA were strikingly similar (Fig 14).

5.3.1.1. Flavonoids in soluble fraction of white teff grain

Peak 1 (t_R 10.49 min, λ_{max} = 268.7, 333.1), had $[M-H]^-$ at m/z 593 (Fig 14). The UV-visible profile matched that of flavone apigenin (authentic standard). Fragmentation produced ions at 503 ($[M-H]^-$ - 90), 473 ($[M-H]^-$ - 120), 383 (aglycone, AGly+113), 353 (AGly+83), 311 (AGly+41) and 283 which are characteristic fragments for di-C-linked glycosides [159] and indicative of the aglycone being apigenin (270 u). A C-linked mono-glycosyl group produces typical fragment ions at AGly+41 and AGly+71 [160] whereas typical fragments for di-C-glycosyl flavones are AGly+83 and AGly+113 [135, 160] (Fig 15B). Further, typical mass losses obtained by cross-link cleavages in pentose and hexose moieties involve ($[M-H]^-$ - 60)/ ($[M-H]^-$ - 90) and ($[M-H]^-$ - 90)/ ($[M-H]^-$ - 120) respectively [135, 148, 160-161] (Fig 15A). Thus, fragments at m/z 503 (593-90) and 473 (593-120) indicate the presence of hexosyl units (162 u). The hexoside moiety attached to flavonoids in most cereal grains are either glucose or galactose sugars [47, 137], with glucose linkage most commonly identified across different cereals [47, 162-163]. Peak 1 was, therefore, identified as apigenin-6,8-C-diglucoside (since 269 ($[M-H]^-$ of apigenin) + 162 + 162 = 593).

Peak 2 (t_R 11.57 min, λ_{max} = 268.7, 336.6) had a similar parent ion mass as peak 1 with $[M-H]^-$ at m/z 593. Fragmentation of peak 2 showed the presence of common ions of mono-C-linked hexoside to apigenin: 341, 311, 283 (Fig 15B). However, another fragment observed in abundance was at m/z 431 ($[M-H]^-$ - 162) which indicates the presence of a O-linked hexoside (since O-linkage produces ion with complete cleavage of the linked molecule) [159]. Also, the abundance of ion at m/z 341 (AGly+71) was lower than 311 (AGly+41) indicating glycosylation at C-8 position vs C-6 position [148]. Peak 2 was thus identified as apigenin-8-C-glucosyl-7-O-glucoside, since the C-7 position is the most common position for glycosylation on a flavone hydroxyl group [161].

Peak 3 (t_R 11.80 min, λ_{max} = 268.7, 336.6) again had similar $[M-H]^-$ at m/z 593 with fragment ions at 341, 311, 283 (Fig 15B) indicating a mono-C-linked hexoside. Another fragment observed had m/z at 323 (AGly+71-18) which indicates a hexoside 2″-O-linked to a hexose C-linked to apigenin [160] (Fig 15C (i)). Further, the m/z 341 (AGly+71) fragment showed high abundance indicating that the hexoside could be C-6-linked (as inferred for peak 2). Thus, peak 3 was identified as apigenin-6-C-glucosyl-2″-O-glucoside. Di-glycosyl apigenin with di-C-linkages or mixture of O&C-linkage (peaks 1, 2 and 3) have been previously identified in wheat [135], and some millets [163].

Peaks 4 and 5 (t_R 12.32&12.46 min, λ_{max} = 268.7, 336.6) had $[M-H]^-$ at m/z 563 with abundant fragment ions at 473 ($[M-H]^-$ - 90), 443 ($[M-H]^-$ - 120), 383 (AGly+113) and 353 (AGly+83) indicating di-C-linkage of a hexose and a pentose moiety (Fig 15 A & B). Further, the ($[M-H]^-$ - 120) fragment showed higher abundance than the ($[M-H]^-$ - 90) fragment in both peaks, likely indicating that the hexose moiety was C-6 linked, as indicated in studies by Ferreres et al [148]. Since the hexose moiety in both peaks were C-6-linked and

in reverse phase chromatrography galactose elutes before glucose [60], the hexoside moiety for peak 4 could be deduced as galactose whereas in peak 5 as glucose. Further, in nature, the common pentose sugars are arabinose or xylose, with conjugation with arabinose typically preferred [60, 164]. Thus, peak 4 was identified as apigenin-6-C-galactosyl-8-C-arabinoside whereas peak 5 was identified as apigenin-6-C-glucosyl-8-C-arabinoside. Similar compounds were identified in bread wheat [47].

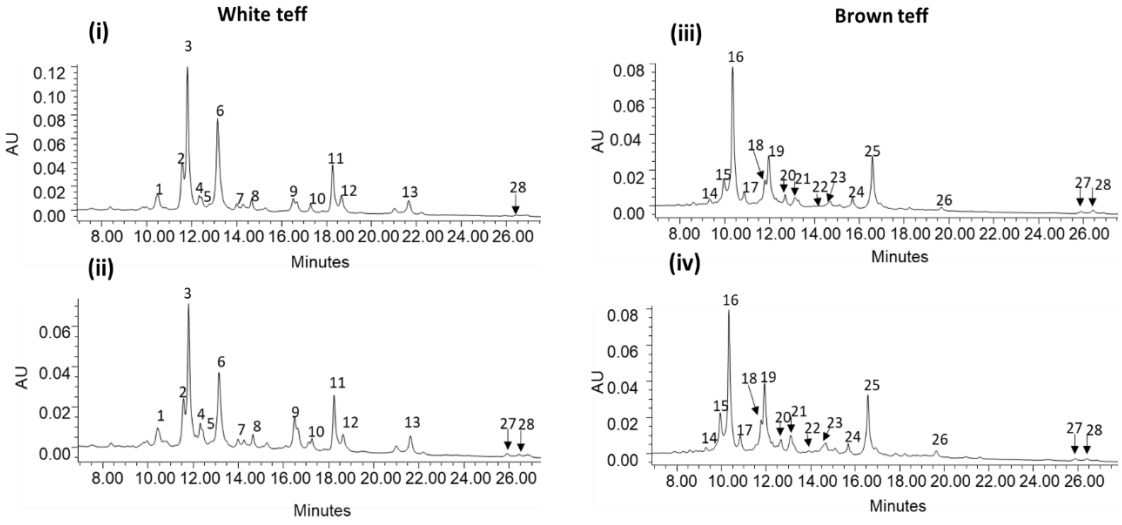
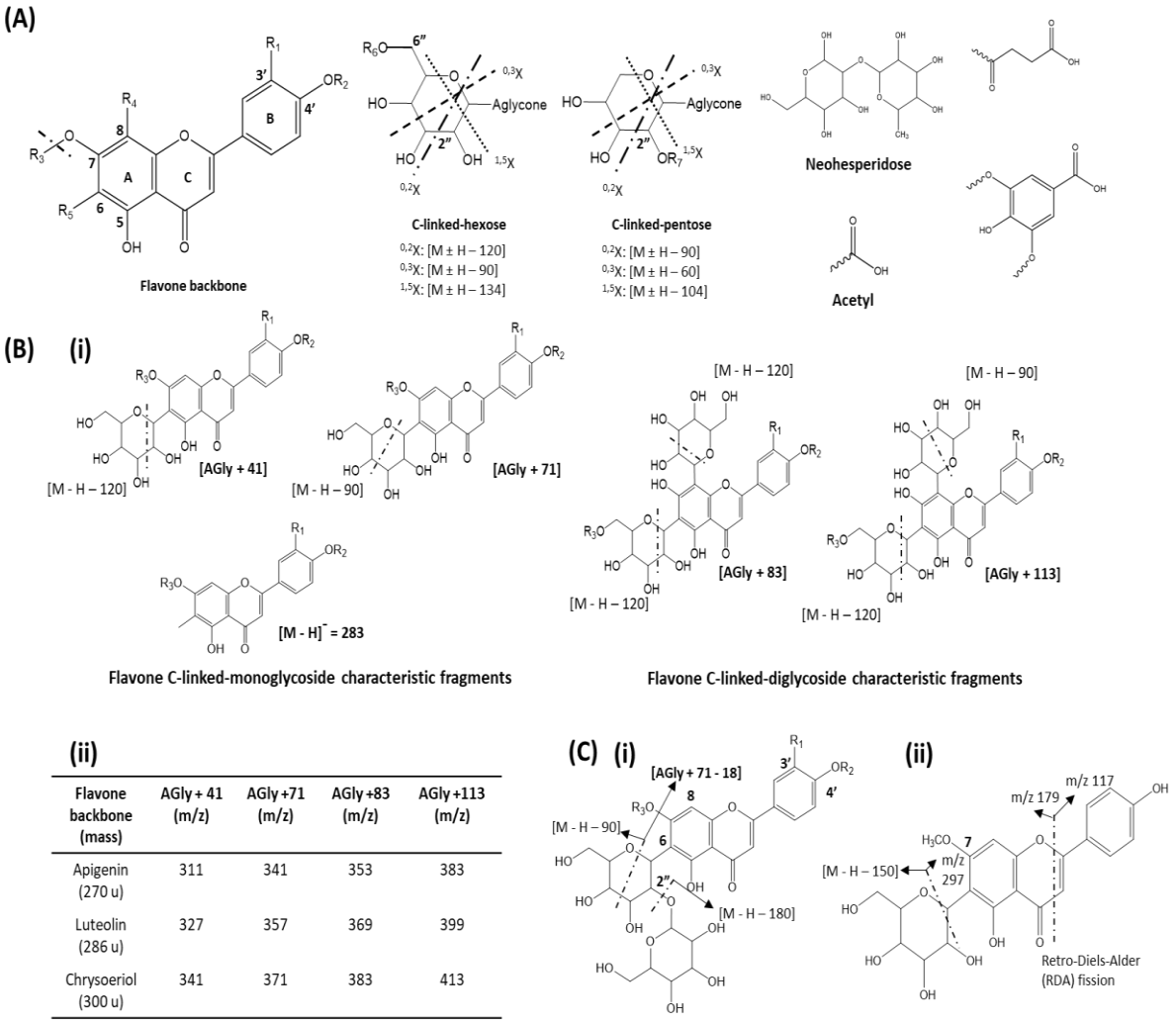


Figure 14: Reverse phase UPLC chromatograms of soluble extracts from (i) White teff (Ethiopia) (ii) White teff (USA) (iii) Brown teff (Ethiopia) (iv) Brown teff (USA) monitored at 325 nm. Peak identities are listed in Table 9. Reprinted with permission from [116].



Flavone backbone (mass)	AGly + 41 (m/z)	AGly +71 (m/z)	AGly +83 (m/z)	AGly +113 (m/z)
Apigenin (270 u)	311	341	353	383
Luteolin (286 u)	327	357	369	399
Chrysoeriol (300 u)	341	371	383	413

Figure 15: Chemical structures of flavone derivatives identified in teff grains (A) Flavone backbone structure with possible O (R_3)/C (R_4/R_5)-linked substitutions (hexoses (glucose/galactose)/pentose (arabinose)/neohesperidose) and acyl group substitutions (acetyl, syringyl, succinyl) as identified in the study with possible positions of acylation at R_6/R_7 of hexose/pentose sugar. Dotted and dashed line indicates typical O and C-linked fragmentation patterns. $R_1 = R_2 = R_3 = H$: Apigenin; $R_1 = OH$, $R_2 = R_3 = H$: Luteolin; $R_1 = OCH_3$, $R_2 = R_3 = H$: Chrysoeriol; (B) (i & ii) Typical characteristic fragments of mono and di-C-linked flavones with typical masses for apigenin, luteolin and chrysoeriol mono and di-C-glycosides (C) (i) Observed fragmentation pattern of 2”-O-hexosyl-C-hexosyl-flavone. If $R_1 = R_2 = R_3 = H$: Apigenin; $R_1 = OH$, $R_2 = R_3 = H$: Luteolin; $R_1 = OCH_3$, $R_2 = R_3 = H$: Chrysoeriol. (ii) Proposed structure with fragmentation pattern for peak 8. Reprinted with permission from [116].

Table 9: Flavones and phenolic acids identified in soluble extract of white and brown teff grains.*

Peak no.	Peak retention time (tR)	Λmax (band II, band I) nm	[M-H]-	MS/MS fragments	Proposed identification
1	10.49	268.7, 333.1	593	503 (2), 473 (27), 383 (65), 353 (100), 311 (89), 283 (3)	Apigenin-6,8-C-diglucoside isomer
2	11.57	268.7. 336.6	593	431 (12), 341 (5), 311 (100), 283 (10)	Apigenin-8-C-glucosyl-7-O-glucoside
3	11.80	268.7, 336.6	593	341 (94), 323 (9), 311(62), 283 (58)	Apigenin-6-C-glucosyl-2”-O-glucoside
4	12.32	268.7, 336.6	563	473 (91), 443 (99.7), 383 (100), 353 (76)	Apigenin-6-C-galactosyl-8-C-arabinoside
5	12.46	268.7, 336.6	563	473 (10), 443 (37), 383 (100), 353 (71)	Apigenin-6-C-glucosyl-8-C-arabinoside
6	13.15	268.7, 336.6	431	341 (6), 311 (96), 283 (100)	Apigenin-8-C-glucoside
7	13.99	268.7, 329.5	431	341 (45), 311 (100), 283 (99)	Apigenin-6-C-glucoside
8	14.65	268.7, 336.6	445	325 (54), 311 (2), 297 (100), 282 (83), 179 (4), 117 (2)	Apigenin-7-O-Me-6/8-C-glucoside
9	16.51	265.1, 336.6	577	269 (100)	Apigenin-7-O-neohesperidoside
10	17.28	268.7, 333.1	773	431 (85), 341 (100), 311 (13)	Apigenin-7-O-(6”-syringyl)glucosyl-6-C-glucoside
11	18.26	268.7, 333.1	743	341 (40), 311 (100)	Apigenin-7-O-(6”-syringyl)arabinosyl-8-C-glucoside
12	18.65	268.7, 333.1	473	341 (7), 311 (100), 283 (39)	Apigenin-8-C-(6”-acetyl) glucoside
13	21.64	268.7, 336.6	515	455 (60), 425 (2), 311 (82), 283 (100)	7-O-methyl apigenin-8-C-(2”-succinyl) arabinoside
14	9.32	265.1, 346.9	609	609 (3), 369 (2), 357 (14), 327 (100)	Luteolin-6,8-di-C-glucoside
15	9.95	268.7, 346.9	609	609 (9), 447 (47), 357 (21), 327 (100)	Luteolin -8-C-glucosyl-7-O-glucoside isomer
16	10.34	268.7, 346.9	609	609 (4), 447 (5), 357 (100), 327 (88)	Luteolin -6-C-glucosyl-7-O-glucoside isomer
17	10.85	268.7, 346.9	755	447 (26), 357 (34), 327 (100)	Luteolin-8-C-glucosyl-7-O-neohesperidoside
18	11.79	268.7, 336.6	593	341 (43), 311 (48)	Apigenin-6-C-glucosyl-2”-O-glucoside
19	11.94	265.1, 346.9	447	357 (34), 327 (100)	Luteolin-8-C-glucoside
20	12.66	265.1, 346.9	623	371 (25), 353 (1), 341 (100)	Chrysoeriol -8-C-(2”-O-glucosyl)glucoside
21	13.11	265.1, 340.1	431	341 (9), 311 (83), 283 (100)	Apigenin-8-C-glucoside
22	14.22	265.1, 343.5	447	285 (100)	Luteolin-7-O-glucoside

Table 9 continued

Peak no.	Peak retention time (tR)	Λmax (band II, band I) nm	[M-H]-	MS/MS fragments	Proposed identification
23	14.69	265.1, 346.9	593	285 (100)	Luteolin-7-O-neohesperidoside
24	15.69	268.7, 346.9	789	609 (5), 357 (100), 327 (63)	Luteolin-7-O-(6”-syringly)glucosyl-6-C-glucoside
25	16.58	265.1, 346.9	759	447 (4), 357 (100), 327 (81)	Luteolin-7-O-(2”-syringyl)arabinosyl-6-C-glucoside
26	19.65	265.1, 346.9	531	411 (5), 357 (3), 327 (100)	Luteolin-8-C-(6”-diacetyl)glucoside
27	25.91	315.2	383	163 (100), 145 (35), 119 (56)	di-coumaroylglycerol
28	26.44	315.2	413	193 (60), 177 (12), 163 (100), 145 (21), 134 (38), 119 (40)	p-coumaroyl-feruloyl-glycerol

* Peaks 1 – 28 have been identified for the first time in white and brown teff soluble extract. Values in parenthesis represent relative signal intensity as % of base peak

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Peaks 6 and 7 (t_R 13.15 & 13.99 min, λ_{max} = 268.7, 336.6/329.5) had $[M-H]^-$ at m/z 431. The MS/MS spectrum showed product ions at m/z 341 (AGly+71), 311 (AGly+41) and 283 indicating a mono-C-linked hexoside (Fig 15B). However, the peaks varied in abundance of the AGly+71 (m/z 341) fragment. Peak 6 showed an abundance of 6%, whereas peak 7 showed an abundance of 45% of the 341 fragment. Since, higher abundance of AGly+71 ion indicates glycosylation at C-6 position [148], peak 6 was inferred as apigenin-8-C-glucoside (vitexin) whereas peak 7 was inferred as apigenin-6-C-glucoside (isovitexin). These compounds have been identified in cereals such as wheat [135, 137], and some millets [163].

Peak 8 (t_R 14.65 min, λ_{max} = 268.7, 336.6) had $[M-H]^-$ at m/z 445. The fragment ions 325 ($[M-H]^-$ - 120) and 311 (AGly+41) indicated the presence of a mono-C-linked hexoside to apigenin. The fragment ion with maximum abundance was at m/z 297 followed by m/z 282 (mass loss of 15 u (methyl group, $-CH_3$)). This indicated the presence of O-linked methyl group to a C-linked-monohexosyl-apigenin. Further, Retro-Diels-Alder fragmentation of the flavonoid apigenin revealed fragments at m/z 179 and 117, indicating that the monohexoside was 6/8-C-linked with the methyl group at C-7-OH on apigenin (Fig 15C (ii)). Thus, based on this, we tentatively identified peak 8 as apigenin-7-O-Me-6/8-C-glucoside.

Peak 9 (t_R 16.51 min, λ_{max} = 268.7, 336.6) had $[M-H]^-$ at m/z 577. MS/MS gave a clear fragment ion at 269 (577-269 = 308 u). The mass loss of 308 indicates the presence of either a C-7-O linked rutinose moiety (L-rhamnopyranosyl-(α1-6)-D-glucopyranose) or a neohesperidose (L-rhamnopyranosyl-(α1-2)-D-glucopyranose) which are two common disaccharides found in association with flavonoids [164]. The absence of partial fragmentation of rhamnoglucosyl moiety led to the conclusion that the mass loss could be associated with

a neohesperidoside [164]. Thus, peak 9 was deduced to be apigenin-7-O-neohesperidoside. This compound has been previously identified in wheat [135].

Peak 10 (t_R 17.28 min, λ_{max} = 268.7, 333.1) had $[M-H]^-$ at m/z 773. Fragmentation of this compound only produced ions at m/z 431 (773-431 = 342 u) and at m/z 341 and 311. Major ion at 431 (269 (apigenin) + 162 (hexosyl)) along with ions at 341 and 311 indicate the presence of C-linked monohexoside (Fig 15B) with the group of 342 u C-7-O-linked. We then hypothesized that the side chain could be a syringic acid linked to hexose (180 (syringly) + 162 (hexosyl) = 342 u) with the likely position of acylation on the sugar at C-6″ as previously reported [60, 162, 164]. Also, since 341 (Agly+71) was the most abundant ion, the hexoside is likely C-6-linked to apigenin. Thus, we tentatively identified this molecule as apigenin-7-O-(6″-syringyl)glucosyl-6-C-glucoside.

Peak 11 (t_R 18.26 min, λ_{max} = 268.7, 333.1) had $[M-H]^-$ at m/z 743. Fragmentation of this compound only produced major ions at m/z 341 and 311 indicating the presence of a mono-C-glycoside linked to apigenin. Like peak 10, the C-linked glycoside was a hexose, which corresponds to a mass of 431 (apigenin (269) + hexosyl (162) = 431). Thus remaining mass loss corresponds to 312 u (since 743- 431= 312) which could be a syringic acid linked to a C-7-O-linked-pentose (since syringly (180) + pentosyl (132) = 312). Also, since m/z 311 was the most abundant ion, the hexoside could likely be linked at C-8 position to apigenin. Further, since the most likely pentose sugar is arabinose [164] we tentatively identified peak 11 to be apigenin-7-O-(6″-syringyl)arabinosyl-8-C-glucoside.

Peak 12 (t_R 18.65 min, λ_{max} = 268.7, 333.1) had $[M-H]^-$ at m/z 473. Fragmentation of this compound produced fragment ions at m/z 341, 311 and 283 indicating the presence of a mono-glycoside linked to apigenin (Fig 15B). Further, 473-acyl-90 = 341 and 473-acyl-

120=311, indicates that the acyl group has a mass of 42 u and the glycoside is a hexoside. Mass of 42 u corresponds to an acetyl group. Also, since the 341 (AGly+71) fragment showed lower abundance compared to 311 (AGly+41) fragment, the hexoside was likely C-8-linked [148]. Thus, peak 12 was identified as apigenin-8-C-(6″-acetyl)-glucoside. Acylation of a flavone with acetyl group has been previously reported in wheat [137].

Peak 13 (t_R 21.64 min, λ_{max} = 268.7, 336.6) had $[M-H]^-$ at m/z 515. MS/MS produced major fragments at m/z 455 ($[M-H]^-$ - 60) and a minor fragment at m/z 425 ($[M-H]^-$ - 90) which indicates the presence of a C-linked pentoside. Further, major fragments at m/z 311 and 283, indicated the presence of mono-C-linked glycoside, likely linked at C-8-position to apigenin. An apigenin-8-C-pentoside would have a mass of 401 (apigenin 269 + pentosyl 132 = 401 u) and thus the remaining mass from parent ion of m/z 515 corresponded to a mass of 114 (515 – 401) which was likely due to succinyl group (100 u) and a methyl group (15 u). Thus peak 13 was tentatively proposed to be apigenin-7-O-Me-8-C-(2″-succinyl)arabinoside as C-2″ is the likely position of acylation on a pentose sugar as indicated by [165]. Acylation with succinyl group has been previously reported in cowpea [60].

5.3.1.2. Flavonoids in soluble fraction of brown teff grain

Peak 14 (t_R 9.32 min, λ_{max} = 265.1, 346.9) had $[M-H]^-$ at m/z 609. The UV-Visible spectrum matched that of flavone luteolin (authentic standard). Fragmentation produced ions at m/z 369 (AGly+83), 357 (AGly+71) and 327 (AGly+41) indicating the aglycone to be di-C-linked to luteolin (aglycone (AGly) mass = 286 u) (Fig 14B). Thus, peak 14 was identified as luteolin-6,8-di-C-glucoside (285 ($[M-H]^-$ of luteolin) + 162 (glucosyl)+162 (glucosyl) = 609).

Peaks 15 and 16 (t_R 9.95 & 10.34 min, λ_{max} = 268.1, 346.9) both had $[M-H]^-$ at m/z 609. Fragmentation produced ions at m/z 447 ($[M-H]^-$ - 162) indicating the presence of a O-linked hexoside, m/z 357 (AGly+71) and 327 (AGly+41) indicating the presence of a mono-C-linked hexoside (Fig 15B). Peak 15 showed most abundant intensity at m/z 327 (AGly+41) fragment whereas peak 16 showed m/z 357 (AGly+71) as most abundant peak. Since C-6-linkage produces higher abundance of (AGly+71) fragment [148], peak 15 was identified to be luteolin-8-C-glucosyl-7-O-glucoside whereas peak 16 was identified to be luteolin-6-C-glucosyl-7-O-glucoside. Compounds 14, 15 and 16 have been previously identified in barley leaves [160].

Peak 17 (t_R 10.85 min, λ_{max} = 268.1, 346.9) had $[M-H]^-$ at m/z 755. Fragmentation produced ions at m/z 447 ($[M-H]^-$ - 308) indicating the presence of a neohesperidose moiety which is C-7-OH linked, as there was absence of partial fragmentation of the rhamnose and glucose moieties (as inferred for peak 9). Further ions detected involved m/z 357 (AGly+71) and 327 (AGly+41) with the base peak at 327 indicating that the glycoside moiety is 8-C-linked to a luteolin. Also, the glycose moiety has to then be a hexose since 285 (luteolin) + 162 (hexosyl) = 447. Thus, peak 17 was identified to be luteolin-8-C-glucosyl-7-O-neohesperidoside. Luteolin-7-O-neohesperidoside has been previously identified in wheat [135].

Peak 18 (t_R 11.79 min, λ_{max} = 268.7, 336.6) had $[M-H]^-$ at m/z 593. The UV-spectrum corresponded to that of apigenin. Fragmentation produced ions at m/z 341 (AGly+71) and m/z 311 (AGly+41) indicating mono-C-linked glycoside of apigenin. Since apigenin (269 (apigenin) + 162 (hexosyl) + 162 (hexosyl) =593) and retention time matched that of peak 3, peak 18 was identified to be apigenin-6-C-glucosyl-2″-O-glucoside.

Peak 19 (t_R 11.94 min, λ_{max} = 265.1, 346.9) had $[M-H]^-$ at m/z 447. MS/MS produced major fragment ions at m/z 357 (AGly+71) and 327 (AGly+41) with m/z 327 as base peak indicating a C-8-glycosyl linkage to luteolin. Since luteolin (285) + hexosyl (162) = 447, peak 19 was identified to be luteolin-8-C-glucoside (orientin).

Peak 20 (t_R 12.66 min, λ_{max} = 265.1, 346.9) had $[M-H]^-$ at m/z 623. Fragmentation produced ions at m/z 371 (AGly+71) and m/z 341 (AGly+41). This indicated that the aglycone has a mass of 300 u which corresponded to chrysoeriol (a 3′-O-methyl form of luteolin). Further, m/z 341 (AGly+41) as base peak indicated the presence of a C-8-linked glycosyl to chrysoeriol. Another fragment at m/z 353 (AGly+71-18) likely indicated that another glycosyl moiety is 2″-O-linked to the 8-C-glycosyl. Peak 20 was then identified to be chrysoeriol-8-C-(2″-O-glucosyl) glucoside (Fig 15C (i)). Similar compounds have been previously identified in *Triticum durum* plants [161] and barley leaves [160].

Peak 21 (t_R 13.11 min, λ_{max} = 265.1, 340.1) had $[M-H]^-$ at m/z 431. Fragmentation produced ions at m/z 341 (AGly+71), m/z 311 (AGly+41) indicating that the aglycone is apigenin with a mono-C-linked hexose, as apigenin (269) + 162 (hexosyl) = 431. Further, the AGly +41 (m/z 311) fragment showed higher abundance than AGly + 71 fragment indicating that the hexoside should be C-8-linked. Also, the retention time was similar to peak 6. Thus, peak 21 was identified as apigenin-8-C-glucoside.

Peak 22 (t_R 14.22 min, λ_{max} = 265.1, 343.5) had $[M-H]^-$ at m/z 447. Fragmentation produced one major ion at m/z 285 (luteolin), indicating the loss of an O-linked hexosyl moiety (447 – 285 = 162 u). Thus, peak 22 was identified as luteolin-7-O-glucoside since C-7-OH is the most common position of glycosylation. This compound has been previously identified in wheat [135], and some millets [163].

Peak 23 (t_R 14.69 min, λ_{max} = 265.1, 346.9) had $[M-H]^-$ at m/z 593. Fragmentation again produced a major ion at m/z 285 (luteolin), indicating the loss of a glucosyl-rhamnose moiety (593 – 285 = 308 u). The loss of 308 u was inferred as neohesperidoside since there was absence of partial fragmentation of the glucose and rhamnose as explained for peak 9. Thus peak 23 was deduced to be luteolin-7-O-neohesperidoside. This compound has been previously identified in wheat [135].

Peak 24 and 25 were identified with similar interpretation as peaks 10 and 11. Peak 24 (t_R 15.69 min, λ_{max} = 268.7, 346.9) had $[M-H]^-$ at m/z 789. Fragmentation produced ions at m/z 609 (789 – 609 = 180) likely indicating the presence of a syringly moiety and major ions at m/z 357 (AGly+71) and 327 (AGly+41) indicating that the aglycone was luteolin with a mono-C-linked glycosyl. Since luteolin (285) + syringly (180) = 465, the remaining mass is 789 – 465 = 324, which corresponds to two hexosyl (162) moieties. Since the fragments obtained only indicated the presence of one C-linked glycoside, the other hexose moiety should be O-linked with likely linkage at the C-7 position. Further, the AGly + 71 fragment showed higher abundance than AGly + 41 fragment thus indicating that the C-linked hexoside was C-6-linked. Peak 24 was thus identified as luteolin-7-O-(6″-syringly)glucosyl-6-C-glucoside.

Peak 25 (t_R 16.58 min, λ_{max} = 265.1, 346.9) had $[M-H]^-$ at m/z 759. Fragmentation produced ions at m/z 447 (759 – 447 = 312). Mass of 312 indicates the presence of syringly (180) group acylated to a pentose sugar (132) linked at C-7 position. Further, other major fragments included m/z 357 (AGly+71) and 327 (AGly+41) indicating again that the aglycone was luteolin C-linked to a hexoside as luteolin (285) + hexosyl (162) = 447. Also, the AGly + 71 fragment showed higher abundance than AGly + 41 fragment, indicating that

the C-linked hexoside was C-6-linked. Thus, peak 25 was identified as luteolin-7-O-(2″-syringyl)arabinosyl-6-C-glucoside.

Peak 26 (t_R 19.65 min, λ_{max} = 265.1, 346.9) had $[M-H]^-$ at m/z 531. Fragmentation produced ions at m/z 411 ($[M-H]^-$ - 120), 357 (AGly+71) and 327 (AGly+41) indicating the linkage of 8-C-hexosyl (since AGly+71 ion was not very abundant) to luteolin (luteolin (285) + hexosyl (162) = 447). The remaining mass of 531 – 447 = 84 u, corresponded to a diacetyl group. Thus, peak 26 was identified as luteolin-8-C-(6″-diacetyl)glucoside. Luteolin with 6″-acetyl-glycoside group has been previously identified in banana passion fruit [159].

5.3.1.3. Phenolic acids in soluble fraction of white and brown teff grain

Peaks 27 and 28 were the only phenolic acid derivatives identified in both white and brown teff soluble extracts (Fig 14). Peak 27 (t_R 25.91 min, λ_{max} = 315.2) had $[M-H]^-$ at m/z 383. UV-visible spectrum and fragmentation ions at m/z 163, 145 and 119 (163 – 44 (COOH)) are indicative of a coumaric acid moiety (authentic standard). The remaining mass loss 383 – 163 = 220 corresponded to another coumaroyl moiety linked to a glycerol. Thus, peak 27 was identified as dicoumaroylglycerol. Glycerol esters of phenolic acids have been previously identified in grains such as sorghum [117].

Peak 28 (t_R 26.44 min, λ_{max} = 315.2) had $[M-H]^-$ at m/z 413. Fragmentation produced ions at m/z 193, 177, 163, 145, 134, 119. Fragments of m/z 163, 145 and 119 correspond to coumaroyl moiety as discussed for peak 28. Fragments of m/z 193, 177 and 134 (193 – 59 (loss of C-COOH unit)) are indicative of a ferulic acid moiety. The remaining mass, 413 – 193 – 163 = 57 indicates a glycerol backbone. Thus peak 28 was identified as p-coumaroyl-feruloyl-glycerol. This compound has been previously identified in sorghum [25].

5.3.1.4. Summary on soluble polyphenol profile of teff grain

Thus, it appears the soluble phenolics in teff grains are mainly flavones; primarily C-glycosides of apigenin and luteolin. It is interesting to note that only apigenin derivatives were present in white teff, whereas, most flavones in brown teff were luteolin derivatives. Thus, the accumulation of flavonoids in white and brown teff grains seems to follow the common flavone biosynthetic pathway, where naringenin is converted to apigenin via flavone synthase. However, the data suggests that only the brown teff perhaps has a functioning flavanone-3′-hydroxylase that converts naringenin to eriodictyol, the primary precursor of luteolin [166]. Interestingly, the profiles of flavonoids from both white and brown teff grains grown in Ethiopia versus those grown in USA were strikingly similar (Fig 14). Kotásková, Sumczynski, Mlček, & Valášek (2016) [158] also reported similarity in the profile of polyphenols in teff grains grown in USA and Bolivia. This suggests environment has little influence on the teff flavonoid biosynthetic pathway.

The information that white and brown teff grains contain different classes of flavones (apigenin vs luteolin) provides a good basis when associating the health outcomes of the different colored grains with phenolic profile in future studies. The genetic basis for the differences observed in flavone composition between white and brown teff grains needs further investigation. We did not detect any anthocyanins or proanthocyanidins in the soluble fractions of brown teff. Thus, a question remained as to what causes the deep reddish-brown color in the grain pericarp of brown teff.

5.3.1.5. Flavone content of white and brown teff grain

The concentration of total flavones in white and brown teff ranged from 1,398 – 2,049 μg/g, db (Table 10). Specifically, the brown teff grains grown in Ethiopia (1,702 μg/g) and USA (1,847 μg/g) were generally similar to the white teff grains grown in Ethiopia (2,049 μg/g) and USA (1,398 μg/g) in total flavone concentration (Table 10). However, as previously noted, white teff only contained apigenin derivatives, whereas brown teff mainly contained luteolin derivatives. In both phenotypes, the diglycoside derivatives were generally the most abundant, accounting for approximately 50% of the flavone content, followed by the monoglycosides and acylated derivatives accounting for approximately 20% each.

Most cereal grains containing flavones as the major flavonoids including millets (fonio, pearl), wheat, and sorghum, have generally much lower flavone content compared to teff. Pearl millet was previously reported to have the highest flavone content of cereal species, with values between 1,000-1,200 μg/g, occurring exclusively as C-glucosyl derivatives of apigenin and luteolin [48]. Other cereal grains generally contain lower levels of flavones, e.g., the total flavones in fonio millet was 500 μg/g [48], in wheat (21-180 μg/g) [47] and sorghum (19 – 386 μg/g) [76, 167].

The high levels of flavones in teff is relevant, because cereal grains are the most important dietary source of flavones. This group of compounds is generally rare in commonly consumed foods, with other important dietary sources being mainly herbs and spices like parsley and thyme [4], which are infrequently consumed in relatively low quantities. Flavones are important because they have structural features that make them among the strongest food-derived anti-inflammatory compounds [15]; additionally cereal derived

Table 10: Concentration (μg/g, db) of identified flavones in soluble extract of white and brown teff grains[a]

Proposed identification	White teff Ethiopia	White teff USA	Proposed identification	Brown teff Ethiopia	Brown teff USA
Apigenin-6,8-C-glucoside isomer	130.8 ± 8.9	121.9 ± 10.9	Luteolin-6,8-di-C-glucoside[c]	43.6 ± 5.1	28.4 ± 5.2
Apigenin-8-C-glucosyl-7-o-glucoside[b]	209.4 ± 12.0	113.6 ± 4.9	Luteolin -8-C-glucosyl-7-O-glucoside [c]	148.6 ± 8.3	190.5 ± 1.1
Apigenin-6-C-glucosyl-2″-O-glucoside[b]	590.4 ± 13.9	375.1 ± 5.7	Luteolin -6-C-glucosyl-7-O-glucoside isomer[c]	599.3 ± 32.9	570.9 ± 10.0
Apigenin-6-C-galactosyl-8-C-arabinoside[b]	66.0 ± 2.5	61.6 ± 3.5	Luteolin-8-C-glucosyl-7-O-neohesperidoside[c]	107.9 ± 9.8	93.3 ± 1.5
Apigenin-6-C-glucosyl-8-C-arabinoside[b]	50.8 ± 4.7	50.3 ± 0.8	Apigenin-6-C-glucosyl-2”-O-glucoside[b]	72.9 ± 6.1	73.0 ± 19.7
Apigenin-8-C-glucoside[b]	388.3 ± 7.4	195.2 ± 3.9	Luteolin-8-C-glucoside[c]	204.8 ± 14.4	250.5 ± 19.4
Apigenin-6-C-glucoside[b]	51.2 ± 3.3	40.4 ± 0.9	Chrysoeriol -8-C-(2”-O-glucosyl)glucoside[c]	79.1 ± 19.3	58.3 ± 3.7
Apigenin-7-O-Me-6/8-C-glucoside[b]	44.7 ± 3.1	34.2 ± 0.3	Apigenin-8-C-glucoside[b]	41.7 ± 2.4	39.6 ± 7.3
Apigenin-7-O-neohesperidoside[b]	64.0 ± 2.8	93.0 ± 2.0	Luteolin-7-O-glucoside[c]	12.8 ± 0.5	23.9 ± 1.5
Apigenin-7-O-(6”-syringyl)glucosyl-6-C-glucoside[b]	52.9 ± 1.9	47.6 ± 1.0	Luteolin-7-O-neohesperidoside[c]	32.2 ± 1.4	69.0 ± 4.3
Apigenin-7-O-(6”-syringyl)glucosyl-8-C-arabinoside[b]	252.4 ± 6.2	168.7 ± 3.6	Luteolin-7-O-(6”-syringly)glucosyl-6-C-glucoside[c]	67.1 ± 4.1	76.1 ± 6.7

Table 10 continued

Proposed identification	White teff Ethiopia	White teff USA	Proposed identification	Brown teff Ethiopia	Brown teff USA
Apigenin-8-C-(6”-acetyl)glucoside[b]	78.4 ± 3.3	43.6 ± 0.9	**Luteolin-7-O-(2”-syringyl)arabinosyl-6-C-glucoside[c]**	288.2 ± 23.5	332.6 ± 21.0
7-O-methyl apigenin-8-C-(2”-succinyl) arabinoside[b]	70.0 ± 1.9	53.0 ± 0.7	**Luteolin-8-C-(6”-diacetyl)glucoside[c]**	22.0 ± 1.6	40.9 ± 3.8
Total flavones	**2049.3 ± 28.8 [A]**	**1398.2 ± 24.6 [C*]**	**Total flavones**	**1720.2 ± 103.5 [B]**	**1847.1 ± 75.8 [B]**

[a]Data are based on UPLC quantification of identified peaks (Table 1) and are expressed as mean ± standard deviation (n=3). [b]As apigenin-7-O-glucoside equivalents. [c]As luteolin-7-O-glucoside equivalents. Peaks that were not structurally identified are not included. Different capital letters indicate significant difference among all the samples in total flavones content based on Tukey’s HSD ($p<0.05$). * indicates significant difference in total flavone content among the growing locations within white and brown teff grains based on Tukey’s HSD ($p<0.05$). Reprinted with permission from [116].

flavones were shown to produce strong synergistic interaction with pulse derived flavonols against inflammation [15], and also protect against estrogen-linked colon carcinogenesis [54]. Thus, teff could be a strategic source of high value bioactive dietary flavones that benefit human health at low levels of use.

5.3.2. Phenolic acids in bound fraction of white and brown teff grain

Only phenolic acids were identified in the bound fractions of teff extracts (Fig 16, Table 11); and no differences in profile between white and brown teff extracts were observed. Peak 29 (t_R 7.11 min, λ_{max} = 308) had $[M-H]^-$ at m/z 163 which corresponded to the mass, retention time and UV spectrum profile of *p*-coumaric acid (authentic standard). Fragmentation produced major ion at m/z 119 (163 – 119 = 44, corresponds to loss of -COOH moiety) which is a characteristic of coumaric acid. Thus, peak 29 was identified as *p*-coumaric acid (Fig 16 B). *p*-Coumaric acid has been identified in bound extract of teff [158].

Peak 30 and 31 (t_R 9.45 & 10.56 min, λ_{max} = 322.3) both had $[M-H]^-$ at m/z 193. The mass, retention time of peak 30 and UV spectrum for both peaks corresponded to that of ferulic acid (authentic standard). Also, both the peaks showed characteristic ions corresponding to that of ferulic acid with m/z at 178 (193 – 178 = 15 indicating the loss of a $–CH_3$ group), 149 (193 – 149 = 44 indicating loss of -COOH group) and 134 (193 – 44 – 15 = 134 indicating the loss of both $-CH_3$ and -COOH groups) [168]. Since the ferulic acid (4-hydroxy-3-methoxycinnamic acid), elutes before the iso-ferulic acid form (3-hydroxy-4-methoxycinnamic acid) on reverse phase HPLC [168], peak 30 was identified as ferulic acid and peak 31 as iso-ferulic acid (Fig 16 B). Ferulic acid was previously identified in the

bound fraction of teff grain [150, 158]. However, this is the first report of iso-ferulic acid in teff grain.

Peaks 32, 33 and 34 (t_R 14.77, 19.59, 20.58 min, λ_{max} = 322.3, 325.9, 325.9) all had $[M-H]^-$ at m/z 385. The UV spectra and masses corresponded to ferulic acid dehydrodimers (DiFA). All 3 peaks showed similar fragments at m/z 326 (385 – 44 – 15 = 326 corresponding to the loss of a -COOH group (44) and $-CH_3$ group (15) respectively, from parent ion), m/z 282 (385 – (2×44) – 15 = 282) and m/z 267 (385 - (2×44) - (2×15) = 267) and other characteristic fragments of ferulic acid at m/z 178, 177, 149 and 134. Only peak 34 showed specific typical fragment ion at m/z 193 with high abundance which could arise from a O-linkage between two ferulic acid monomers. Thus matching fragmentation pattern, retention time and UV spectrum profile of the 3 peaks with previous studies (Guo & Beta, 2013), peak 32 was identified as 8,5'-DiFA, peak 33 as 5,5'-DiFA and peak 34 as 8-O-4'-DiFA (Fig 16C i, ii, iii). Ferulic acid dehydrodimers have been previously identified in several grains such as barley, wheat, maize, oats, millets [163, 168].

Thus, the bound fraction mainly consisted of 6 phenolic acids (4 of them identified for the first time in teff) dominated by monomeric ferulic acid. The profile of bound phenolics generally agreed with those reported in other cereal grains such as barley, yellow corn, wheat, red rice and oats [168]. No flavonoids, anthocyanins or proanthocyanidins were detected in the bound fraction. The accumulation of similar profile of phenolic acids in white and brown teff grains indicates similar biosynthesis of the compounds in the grains.

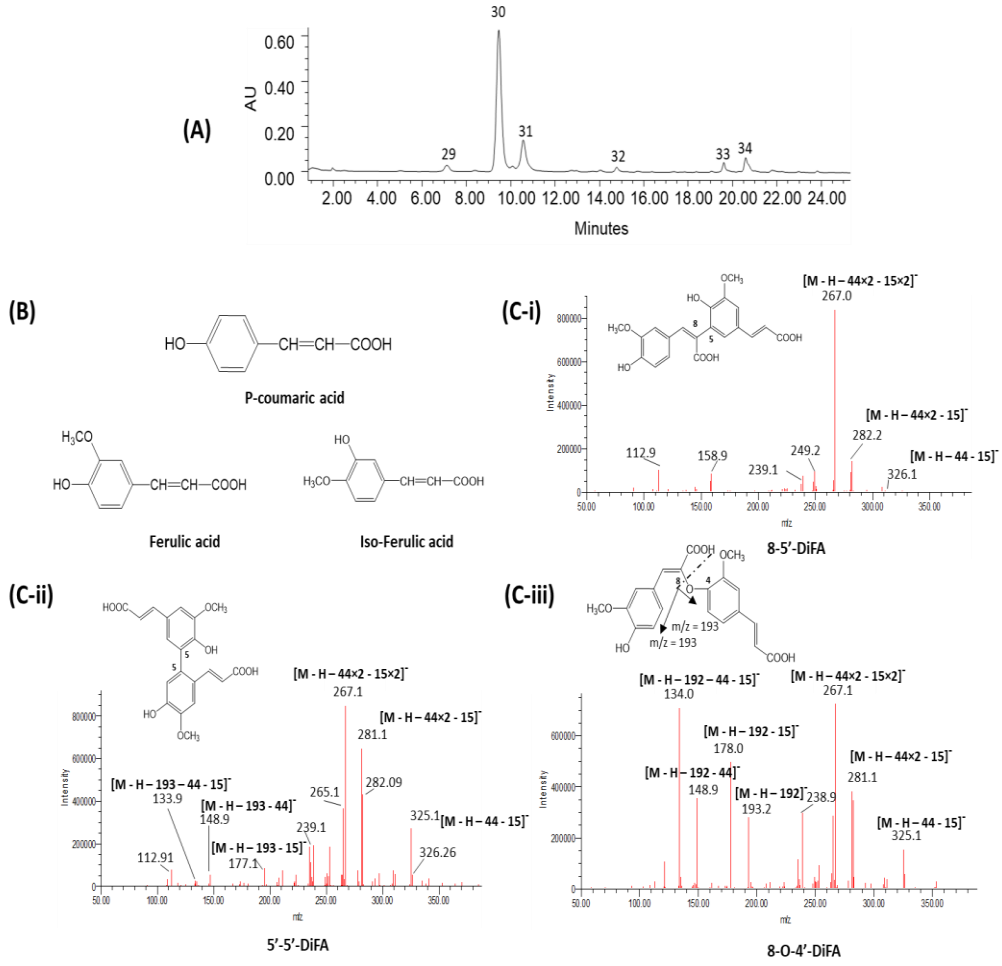

Figure 16: (A) Reverse phase UPLC chromatogram of bound extracts from white and/or brown teff grains monitored at 325 nm. Peak identities are listed in Table 3. (B) Chemical structures of monomeric phenolic acids identified in bound extract (C-i,ii,iii) MS/MS spectra and structures of identified ferulic acid dehydrodimers at CV = 30, CE = 30. Reprinted with permission from [116].

5.3.2.1. Phenolic acids content in bound fraction of teff grain

The phenolic acid content in the bound fraction of different teff grains are presented in Table 11. Total content of bound phenolics did not vary significantly between white and brown teff grains or between the grains grown in Ethiopia and USA. The total bound phenolic acid was in the range of 600-728 µg/g, db of grain. The monomeric forms (p-

coumaric acid and ferulic acids) accounted for approximately 82% of the total bound phenolics, and the ferulic acid dehydrodimers accounted for the remainder. The most abundant phenolic acid was monomeric ferulic acid (350-475 µg/g of grain), followed by iso-ferulic acid (80-100 µg/g). Among the ferulic acid dehydrodimers, 8-O-4′-DiFA was the most abundant form (64-80 µg/g). The quantitative profile of the monomeric phenolic acids generally agreed with Shumoy & Raes, (2016) [150] and Kotásková, Sumczynski, Mlček, & Valášek, (2016) [158] who identified the monomeric ferulic acid to be the most abundant phenolic acid in the bound fraction of extracts from white and brown teff grains. Thus, phenolic acids in teff were mainly in the bound fraction, as is the case for other cereals.

Table 11: Identification and quantification[b] (µg/g, db) of phenolic acids in bound extract of white and brown teff grains.

Peak no.	Peak retention time (tR)	λmax	[M-H]-	MS/MS fragments	Proposed identification	White teff Ethiopia	White teff USA	Brown teff Ethiopia	Brown teff USA
29[a]	7.11	308	163	119 (100)	p-coumaric acid	22.0 ± 2.8	18.6 ± 3.3	22.6 ± 2.3	19.2 ± 3.6
30[a]	9.45	322.3	193	178 (8), 149 (2),134 (100)	Ferulic acid	455.0 ± 56.8	475.0 ± 50.2	383.9 ± 40.2	350.4 ± 24.6
31	10.56	322.3	193	178 (6), 149 (2), 134 (100)	Iso ferulic acid[c]	92.2 ± 13.4	100.5 ± 10.4	79.9 ± 13.0	86.6 ± 15.0
32	14.77	322.3	385	326 (1), 282 (17), 267 (100)	8,5'-DiFA[c]	22.2 ± 1.8	21.9 ± 4.3	24.6 ± 2.5	21.1 ± 4.0
33	19.59	325.9	385	326 (6), 325 (31), 282 (49), 267 (100), 177 (2), 134 (3)	5,5'-DiFA[c]	34.3 ± 6.0	39.8 ± 5.2	51.5 ± 4.1	44.8 ± 13.9
34	20.58	325.9	385	326 (8), 325 (23), 282 (47), 267 (100), 193 (38), 178 (69), 149 (48), 134 (97)	8-O-4'-DiFA[c]	64.1 ± 11.6	72.6 ± 17.9	80.9 ± 7.5	78.5 ± 20.9
					Total	**689.7 ± 92.4**	**728.3 ± 91.3**	**643.4 ± 69.6**	**600.6 ± 82.1**

[b]Data are based on UPLC quantification of identified peaks and are expressed as mean ± standard deviation (n=3). [c]As ferulic acid equivalents.

[a] Previously identified in teff. Values in parenthesis represent relative signal intensity as % of base peak. Reprinted with permission from [116].

5.3.3. Evidence for the presence of proanthocyanidins in brown teff grain

Acid catalyzed thermal hydrolysis of extracted residue of white and brown teff grains in acidified methanol produced a characteristic red colored extract, presumptive positive for presence of condensed tannins (proanthocyanidins), in the brown but not white teff. UPLC analysis of the red solution showed the presence of a single peak (Fig 17); the peak had UV-vis spectrum, λ_{max} = 524.9 nm, and retention time corresponding to that of cyanidin (authentic standard). Further, MS/MS analysis revealed that the peak had parent ion $[M+H]^+$ at m/z 287 and fragmentation ion with highest intensity at m/z 137, and other major fragments at m/z 139, 143, 165, 175, 213, 231, similar to cyanidin. Thus, the compound was positively identified as cyanidin.

A known reaction is release of anthocyanidins from their respective proanthocyanidins by acid catalyzed hydrolysis and oxidation in the presence of alcohol and heat [136]. The release of only one anthocyanidin, cyanidin, from the brown teff residue thus indicates the presence of only procyanidins (condensed tannins exclusively containing catechin and/or epicatechin as the chain units). Thus, it can be concluded that brown teff contains procyanidins, but these compounds are lacking in white teff. Furthermore, the brown teff procyanidins appear to be present in a form that is largely unextractable, similar to the proanthocyanidins reportedly responsible for red wheat pericarp color [55]. Whether the unextractable tannins have a significant impact on nutritional properties of brown teff, as well as the genetic basis for their synthesis should be investigated.

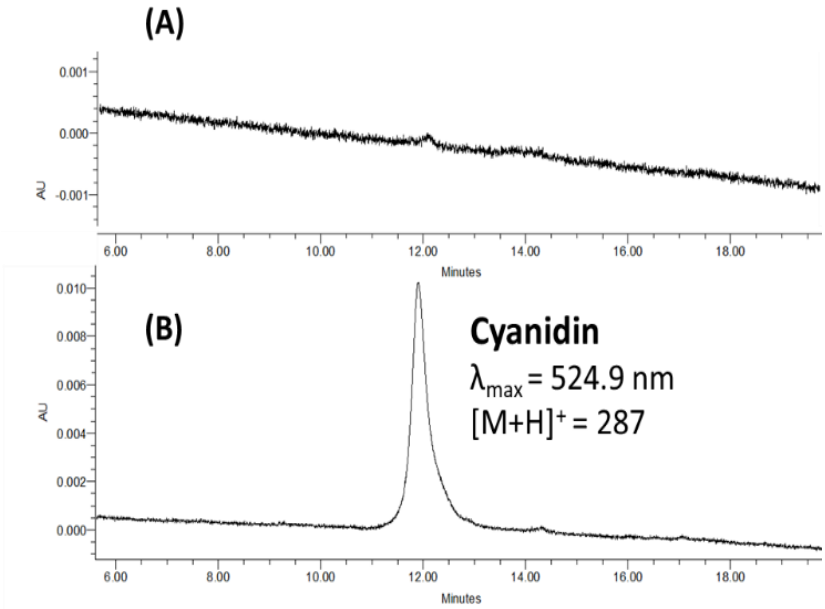

Figure 17: Reverse phase UPLC chromatogram of acid hydrolyzed extracts from (A) white and (B) brown teff grains monitored at 520 nm and MS/MS analyzed in positive ion mode. Reprinted with permission from [116].

5.4. Conclusions

It appears the extractable phenolic compounds in teff grain are almost exclusively flavones, primarily their C-glycosides. Distinct differences exist between flavone profile of white versus brown teff, where white teff contains only apigenin glycosides, whereas brown teff contains mostly luteolin glycosides; this indicates key differences in their flavonoid biosynthetic pathways. Surprisingly though, the overall flavone contents were similar between the red and white teff. Furthermore, the bound fractions of the white and brown teff extracts contained similar profiles and levels of phenolic acids. Thus, the differences in pericarp color between white and brown teff cannot be attributed to phenolic profile obtained by standard protocols. Using microwave energy to catalyze acid-induced thermal hydrolysis and oxidation of unextractable residues, we demonstrated that condensed tannins are present

as procyanidins in unextractable form in the brown, but not white, teff. This is the most likely explanation for the dark pericarp color of the brown teff. Our data indicates that teff grains contain particularly high levels of flavones, much higher than reported in other cereal grains, or other food commodities. This renders teff a valuable source of the relatively rare dietary flavones; and also an excellent model to investigate the bioactive properties of cereal flavones in a natural food matrix. The nutritional and health implications of the unextractable tannins in brown teff warrant further studies.

6. EFFECT OF FERMENTATION AND BAKING ON STRUCTURAL PROFILE OF POLYPHENOLS AND ANTIOXIDANT POTENTIAL OF WHOLE GRAIN CEREALS

6.1. Introduction

Several epidemiological studies have shown that consumption of whole grain cereals and pulses is strongly associated with reduced risk of chronic diseases [7-8]. Several factors contribute to the health beneficial outcomes from consumption of whole grains, among which polyphenols, and more specifically flavonoids are gaining increased attention in recent years [53]. Flavonoids in grains are typically conjugated with one or more sugar residues (e.g. glucose, galactose) linked to hydroxyl groups (O-linkage) or directly linked to an aromatic carbon atom (C-linkage) on the flavonoid molecule [46, 53]. Pulses (e.g. cowpea, dry beans, lentils) mainly contain flavonol glycosides that are O-linked to the flavonoid backbone. On the other hand, most of the commonly consumed cereals such as wheat, and millets such as teff mainly contain C-linked flavone glycosides (mainly apigenin-C-glycosides) [53, 116, 135]. Sorghum is unique in that, different varieties may also contain the aglycone form of 3-deoxyflavonoids (flavones, flavanones) and their respective O-glycosides. Other polyphenols present in cereals and pulses include phenolic acids either in free form, or as O-glucosides and/or glycerol ester derivatives [142, 169].

Several processing methods are typically employed to transform whole grains into consumable products, among which fermentation is used as a traditional technique in several cultures around the world. Common traditional whole grain fermented products include *Injera*, made from whole grain teff in Ethiopia, [68], wheat and rye in (sourdough) bread [69] and several pulses are fermented into snack or breakfast products in countries like India,

Indonesia, Sri Lanka, Nigeria and in regions in West and Central Africa [23]. Traditionally, the main purpose of fermentation was to extend shelf life and improve the flavor and nutritive value of foods [23-24].

Recent studies have shown fermentation to offer additional advantages with respect to whole grains and their related products. For example, fermentation is gaining popularity in gluten free applications due to studies showing their ability to partially hydrolyze gliadin peptides (the protein responsible for celiac disease) [70]. Further, the process shows added advantages in improving texture of gluten free products due to release of microbial exopolysaccharides, replacing the use of gums [70-71]. Also, in the process of natural fermentation, bacteria and yeasts produce several enzymes that can release bound phenolics (due to esterase activity) and/or induce structural changes to flavonoids, especially the O-glycoside forms (due to glucosidase activity) and metabolize phenolic acids. This could directly affect the bioaccessibility, bioavailability and hence bioactivity of flavonoids from fermented grain products [25]. However, there are no studies specifically evaluating the changes with respect to aglycone and C-linked flavonoid glycosides (especially di-C-linked, and O-and-C-linked) forms upon fermentation and heat treatment.

This work thus evaluates the changes in flavonoid profile upon fermentation and subsequent heat treatment (baking) of grains typically used to make sourdough products yet have distinctly different phenolic profiles. White sorghum was chosen to be a model for flavone aglycones (apigenin, luteolin) and phenolic acids, lemon yellow sorghum to be a model for O-linked glycosyl-flavonoids, wheat to be a model for di-C-linked-glycosyl-flavonoids and teff was a model containing a mixture of mono and di-C-linked-glycosyl and

O-linked-glycosyl-flavonoids, based on their abundances in the respective grains (as demonstrated in Chapters 4 and 5).

Baking was employed as a common thermal processing technique utilized for producing fermented grain-based products (e.g. pancakes, sourdough breads). Pancakes are a quick bread and are a simple model to evaluate the effect of heat on a fermented dough. Also, fermented grain based pancake like products are popular in several cultures around the world, such as *Injera* in Ethiopia, *Dosa* in India, *Kisra* in Sudan, *Hopper* in Sri Lanka [170] and sourdough based pancakes are gaining popularity in the US (mimicking buttermilk pancakes to provide the tartness and/or to reduce gluten intolerance). Thus, the pancake provides a good model to evaluate effect of fermentation and heat in a food matrix. Outcomes of this work could help better understand the effect of fermentation and subsequent baking on different polyphenol forms which could influence their bioavailability and potential bioactivity upon consumption of fermented products made from whole grains.

6.2. Materials and methods

6.2.1. Plant materials

Three different cereal grain types wheat, teff, and sorghum, characterized by differences in flavonoid profile were used in the study. Hard white wheat was obtained from Naturals Wheat (Kansas, USA). Red wheat (TAM 231-8) was obtained from Texas A&M AgriLife Research, lemon yellow sorghum (ATX-642) and white sorghum (ATX 635-RTX 436) were grown and harvested in College Station, TX (2016 and 2011, respectively). White and brown teff grains grown in Idaho, USA (Shiloh farms, PA, USA) in 2016 were commercially

purchased. The whole seeds were ground using UDY mill to pass through a 1 mm mesh sieve and stored at -20 ºC until used.

6.2.2. Chemicals and reagents

All reagents were analytical grade. Apigenin-7-O-glucoside, luteolin-7-O-glucoside, eriodictyol and its 7-O-glucoside were purchased from Extrasynthese (Genay Cedex, France), naringenin was from MP Biomedicals (Solon, Ohio), ferulic acid was from Indofine (Hillsborough, NJ, USA) and gallic acid, p-coumaric acid, caffeic acid, ethanolamine, 2,2'-Azobis(2-methyl-propionamidine)dihydrochloride (AAPH) was from Sigma-Aldrich (St. Louis, MO, USA). Folin Ciocalteu reagent was from MP biomedicals (Solon, Ohio, USA), potassium persulfate was from Mallinckrodt Chemicals (NJ, USA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was from TCI (Tokyo Chemical Industry, OR, USA).

6.2.3. Fermentation and thermal treatment

Process of fermentation and subsequent baking was followed according to Shumoy et al. [68] with slight modifications. Briefly, ground samples were mixed with distilled water in a 1:2 proportion to form the dough and allowed to naturally ferment for up to 96 h incubated at 30 °C. Fermented dough samples were collected at 48 h and 96 h and immediately frozen at -20 °C. Further, dough at each time point (48 h, 96 h), was baked on a skillet at 177 °C into pancakes, 4 min on each side, cooled to room temperature and frozen at -20 °C. Non-fermented dough (0 h) and pancake samples made from non-fermented dough (0 h) were included as controls. pH of the dough was measured at each of the time points (0 h, 48 h and 96 h). All samples were then freeze dried, ground using UDY mill to pass through a 1 mm mesh sieve and stored at -20 ºC until used.

6.2.4. Extraction of soluble polyphenols

Extraction of soluble polyphenols was followed according to Ravisankar et al. (2018) [116]. Briefly, the ground samples were extracted with 80% methanol (1:5 flour:solvent, w/v) for 2 h with shaking at room temperature. The extracts were centrifuged (10,000 g for 10 min) using a Heraeus Megafuge 11R Centrifuge (Thermo Fisher Scientific, Asheville, NC) at 20 ºC and the supernatant was transferred to new set of tubes. The residue was further extracted twice (1:2.5 residue:solvent (80% methanol), w/v) each time for 30 min and centrifuged. The supernatants were combined and stored at -80 ºC until further use.

6.2.5. UPLC-Electrospray Ionization (ESI)-MS/MS analysis

Identification and quantification of phenolic acids and flavonoids in the grains was performed according to Ojwang et al. (2012) [60] and Ravisankar et al. (2018)[116] with slight modifications on a Waters-ACQUITY-UPLC-TQD-MS/MS system (Waters Corp., Milford, MA) equipped with a photodiode array eλ detector and interfaced with a mass spectrometer equipped with a tandem quadrupole (TQD) electrospray ionization (ESI) detector. The separation was performed on a Kinetex C18 column (100 × 2.10 mm, 2.6 µm) (Phenomenex, Torrance, CA) at 40 °C with flow rate of 0.4 mL/min: The mobile phases consisted of 0.05% formic acid in water (solvent A) and 0.05% formic acid in acetonitrile (solvent B). The percentage of solvent B: 0-5 min 5% B, 5-35 min 35% B, 35-45 min 70% B, 45-50 min 70% B, 50-53 min 5% B and 53-60 min 5% B for analysis of polyphenols from teff grains and 0-2 min 5% B, 2-8 min 20% B, 8-15 min 70% B, 15-20 min 70% B, 20-23 min 5% B and 23-27 min 5% B for analysis of polyphenols from wheat and sorghum. For MS/MS analysis, the phenolic extracts were filtered using a syringe filter with a 0.22 µm PTFE membrane and injection volume of 2 µL was used, whereas for quantification, the combined supernatants

were injected at a volume of 5 μL. Flavones were monitored at 340 nm, flavanones at 280 nm, phenolic acids at 325 nm and 280 nm and metabolites were scanned over 200 – 520 nm. Mass spectrometric data was acquired in negative mode for all compounds. The source, ionization gas flow and data processing conditions was similar to that reported by Ojwang, Dykes, & Awika, 2012[60]. The MS scan was recorded in the range of 100–1000 Da. Parent ion scanning mass parameters was optimized as follows: Capillary voltage was 3 kV; and cone voltage was set at 30 V for negative ionization respectively. The MS/MS scan was optimized as follows: cone voltage of 30 V and collision energy of 15-40 V. Compound identification was done based on matching UPLC retention profile, UV–vis spectra and MS data with authentic standards. Where standards were not available, compounds were identified based on the fragment patterns compared with reports in literature.

Quantification of the compounds was done by interpolating peak areas based on standard curves of corresponding pure compounds. In the case where no standards were available, quantification was done based on the assumption that their molar absorptivity is similar to those of the closest corresponding available compounds similar to the procedure followed by Ojwang, Dykes, & Awika, 2012 [60]. Data was reported on dry basis, based on three separate runs.

6.2.6. Determination of extractable phenol content

Extractable phenol content was determined in the extracted samples by Folin-Ciocalteu assay as previously described by Kaluza et al 1980 [171] using gallic acid as standard and sample extracts diluted appropriately to fit the range of the standard curve. Briefly, 0.1 mL of sample in 1.1 mL distilled water was reacted with 0.4 mL Folin reagent and 0.9 mL 0.5

M ethanolamine for 20 min at room temperature. The absorbance of the samples was then measured using a UV-visible spectrophotometer (UV-2450, Shimadzu, Kyoto, Japan) at 600 nm against a reagent blank. The phenol content was expressed as mg of gallic acid equivalents/g of sample (mg GAE/g) on dry weight basis based on calibration curve of gallic acid.

6.2.7. Trolox equivalent antioxidant capacity (TEAC) assay

The end point TEAC assay as previously described by Awika et al 2003 [172] was used to measure ABTS radical scavenging activity of extracts which were appropriately diluted to fit the linearity range of trolox standard curve (0-1000 μM). Briefly, a 1:1 mixture of ABTS (8mM) and potassium persulfate (3mM) in distilled water was reacted in dark for 16 h (stock). 2.9 mL of ABTS working solution (stock diluted 1:30 times with PBS (pH 7.4) to an absorbance of 1.5 at 734 nm) was reacted with sample (0.1 mL) for 30 min. The absorbance was then measured at 734 nm and radical scavenging activity was expressed as μmol trolox equivalents (TE)/ g of sample on dry weight basis based on trolox standard curve.

6.2.8. Oxygen radical absorbing capacity assay (ORAC)

The kinetic based ORAC method was used to measure the antioxidant activity of extracts according to method previously described by Awika et al 2003 [172] against a trolox standard curve. Prior to analysis, extracts were appropriately diluted to fit the linearity range of Trolox standard curve. Competition of sample extracts/trolox with peroxyl generator 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH 153 mM in PBS) in protecting fluorescence of sodium fluorescein (4× 10-3 μM in phosphate saline buffer (pH 7.4) was measured at excitation 485 nm and emission 528 nm using plate reader (Biotek Synergy HT

plate reader with automatic dispenser (Biotek, Winooski, VT). The net area under the curve (AUC) was determined by subtracting that of the sample from the blank and the ORAC capacity is expressed as μmol trolox equivalents (TE)/ g of ample on dry weight basis based on net AUC of trolox standard curve.

6.2.9. Statistical Analysis

Three replications of each treatment were performed. Analysis was done using JMP pro 12 (Version 12.0.1, SAS Institute, Inc., Cary, NC) with one-way Analysis of Variance (ANOVA). Post Hoc test (Students-t-test/Tukey-Kramer HSD) was used to compare treatments means. Significance levels were defined using $p < 0.05$.

6.3. Results and discussion

6.3.1. Acidity of fermented dough

In general, across all grain samples, the initial pH of the unfermented dough ranged between 6.05-6.37. After fermentation for 48 h, the dough pH dropped down to 3.71-4.55 and further fermentation to 96 h, dropped the pH to 3.39-3.98. This pH drop confirmed the occurrence of fermentation as there is a general increase in organic acids such as lactic, acetic and propionic acids produced by lactic acid bacteria and yeast upon grain fermentation as observed in previous studies [25, 68]. Thus, the micro-organisms dominating the natural fermentation employed in this study could mainly be lactic acid bacteria and yeast.

6.3.2. Identification of phenolic acid metabolites and bound phenolics released in the process of fermentation in wheat, sorghum and teff dough and pancakes

Peak 43 (t_R 5.996 min, λ_{max} = 318.8) had $[M-H]^-$ at m/z 195. The UV-visible spectra and MS/MS fragment ions at 136 and 121 matched that of dihydroferulic acid. This was previously identified as a fermentation metabolite of ferulic acid produced by the action of *L. plantarum* and *L.fermentum* as identified in Svensson et al. (2010) [25] due to enzyme reductase activity [173] (Fig 18A). This compound was identified in the fermented white sorghum dough and pancake samples (Figs 19& 20).

Peak 45 (t_R 7.188 min, λ_{max} = 258, 297.3) had $[M-H]^-$ at m/z 135 (Figs 19& 20). The parent mass and fragment ion at m/z 89 matched that of vinyl catechol as identified in Svensson et al. 2010 [25]. This compound is a lactic acid bacteria metabolite of caffeic acid produced due to their phenolic acid decarboxylase activity [25, 173] (Fig 18B). This compound was identified and increased in abundance in the fermented white sorghum dough and pancake samples and was absent in the non-fermented samples (Figs 19 & 20).

Peak 60 and 61 (t_R 17.359 and 14.914 min, λ_{max} = 272.3, 282 and 279.4) had $[M-H]^-$ at 325 and 297 respectively. Both the compounds had base peak at m/z 183 and other abundant ions at m/z 197, 169, 119. UV-visible spectrum and major ion at m/z 183 with another fragment at m/z 169 match that of a methylgallate as identified by Monagas et al. 2005 [174]. For peak 60, $[M-H]^-$ - 197 (observed fragment) = 100 amu, which could likely indicate a succinoyl group being attached to methylgallate. Further, 197 – 183 = 14, which indicates the presence of another methyl group on the molecule of peak 60. Mass of peak 61 ($[M-H]^-$ at 325) – mass of peak 61 ($[M-H]^-$ at 297) gives a difference of 28 amu which corresponds to an extra ethyl group (C2H4) on peak 61. Two studies by Olschlager et al. (2008), Olschlager et al. (2010),

found the presence of procyanidins and propelargonidins containing 3-O-methylgallates and/or 3-O-(3,4-dimethylgallate) on their backbone in buckwheat grains [175-176], thus indicating the likely presence of dimethyl gallate in grains. In this study, the methylgallates were identified across all fermented grain samples and hence could be bound to cell wall and released in the process of fermentation. Thus, tentatively, both peaks 60 and 61 were identified as methylgallate ester derivatives with the proposed structures as indicated in figure 18C : Peak 60 –succinoyl-dimethylgallate and Peak 61 – Ethyl-Succinoyl-dimethylgallate. The dimethylgallates could have undergone further transformation with acyl/ethyl groups (being released in the process of fermentation) in the fermented samples. Both the peaks were present and increased in abundance in fermented wheat and teff dough and pancake samples whereas only peak 60 was present in sorghum fermented samples. These peaks were absent in all unfermented treatments (Figs 19, 20, 21, 22, 23, 24) .

All other peaks were present in unfermented wheat, sorghum and teff grains and are described in Chapters 4 & 5.

Table 12: Metabolites and bound phenolic acids identified in soluble extract of wheat, sorghum and teff fermented dough and pancake samples (48 h and 96 h).

Peak no.	Peak retention time (tR)	λmax	[M-H]-	MS/MS fragments	Proposed identification
43	5.996	283	195	136 (9), 121 (100)	Dihydroferulic acid
45	7.188	258, 297.3	135	89 (100)	4-vinylcatechol
60	14.914	279.4	297	197 (3), 183 (100), 119 (20)	Succinoyl-dimethylgallate
61	17.359	272.3, 282	325	197 (2), 183 (100), 169 (4), 119 (2)	Ethyl-succinoyl-dimethylgallate

Values in parenthesis represent relative signal intensity as % of base peak.

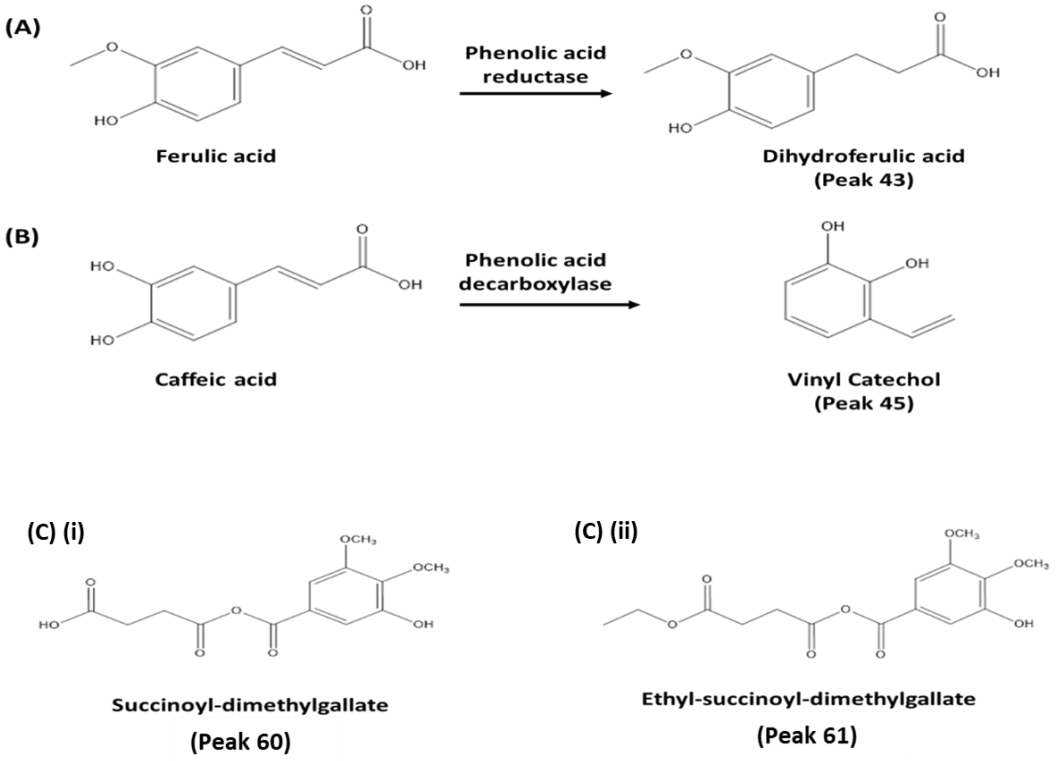

Figure 18: Phenolic acid metabolites and bound phenolic acids released in the process of fermentation. (A) Dihydoferulic acid, produced by the action of bacterial enzyme phenolic acid reductase on ferulic acid; (B) vinyl catechol produced by the action of bacterial enzyme phenolic acid decarboxylase on caffeic acid; (C)(i) Proposed structure for peak 60 – succinoyl-dimethylgallate (C)(ii) Proposed structure for peak 61 – ethyl-succinoyl-dimethylgallate (Table 12).

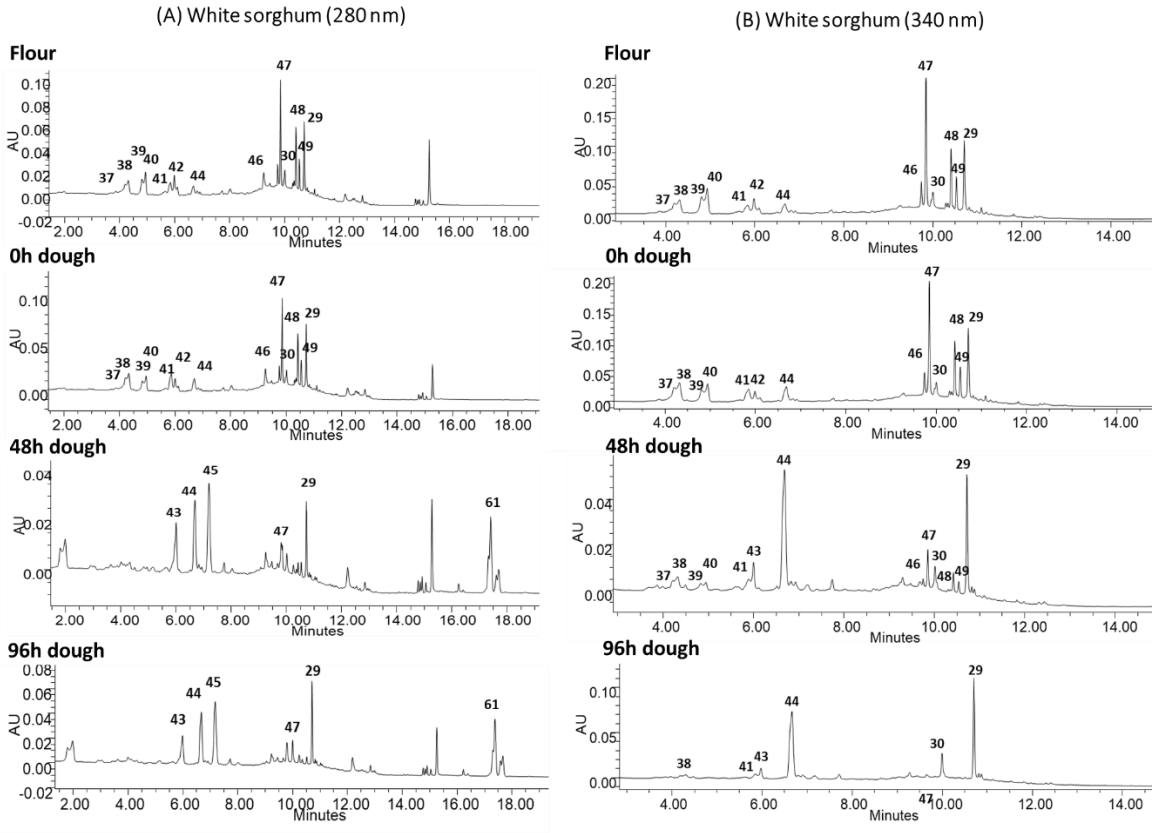

Figure 19: Effect of fermentation on UPLC profile of polyphenols identified in white sorghum at 280 nm (A) and 340 nm (B). Non-fermented dough = 0 h; and fermented doughs = 48 h and 96 h. Peak identities are as described in Section 4.3.2.1. Peak numbers 37-42, 44, 46-49 correspond to peak numbers 1-11 in Table 3 and peak numbers 29, 30 correspond to peak numbers 12, 13 in Table 4. Other peak identities are as described in Section 6.3.2, Table 12.

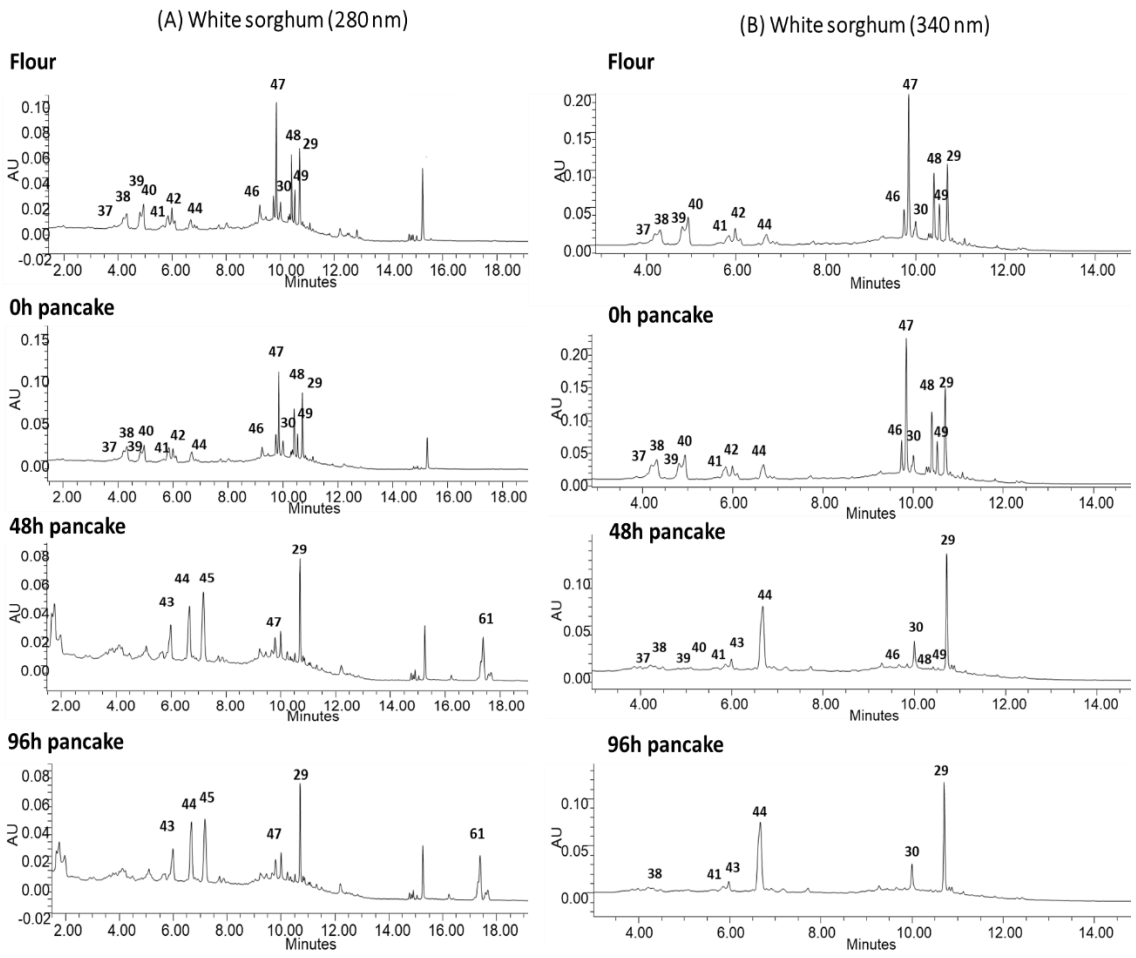

Figure 20: Effect of baking on UPLC profile of polyphenols identified in white sorghum pancakes at 280 nm (A) and 340 nm (B). Non-fermented pancake = 0 h; and fermented pancakes = 48 h and 96 h. Peak identities are as described in Section 4.3.2.1. Peak numbers 37-42, 44, 46-49 correspond to peak numbers 1-11 in Table 3 and peak numbers 29, 30 correspond to peak numbers 12, 13 in Table 4. Other peak identities are as described in Section 6.3.2, Table 12.

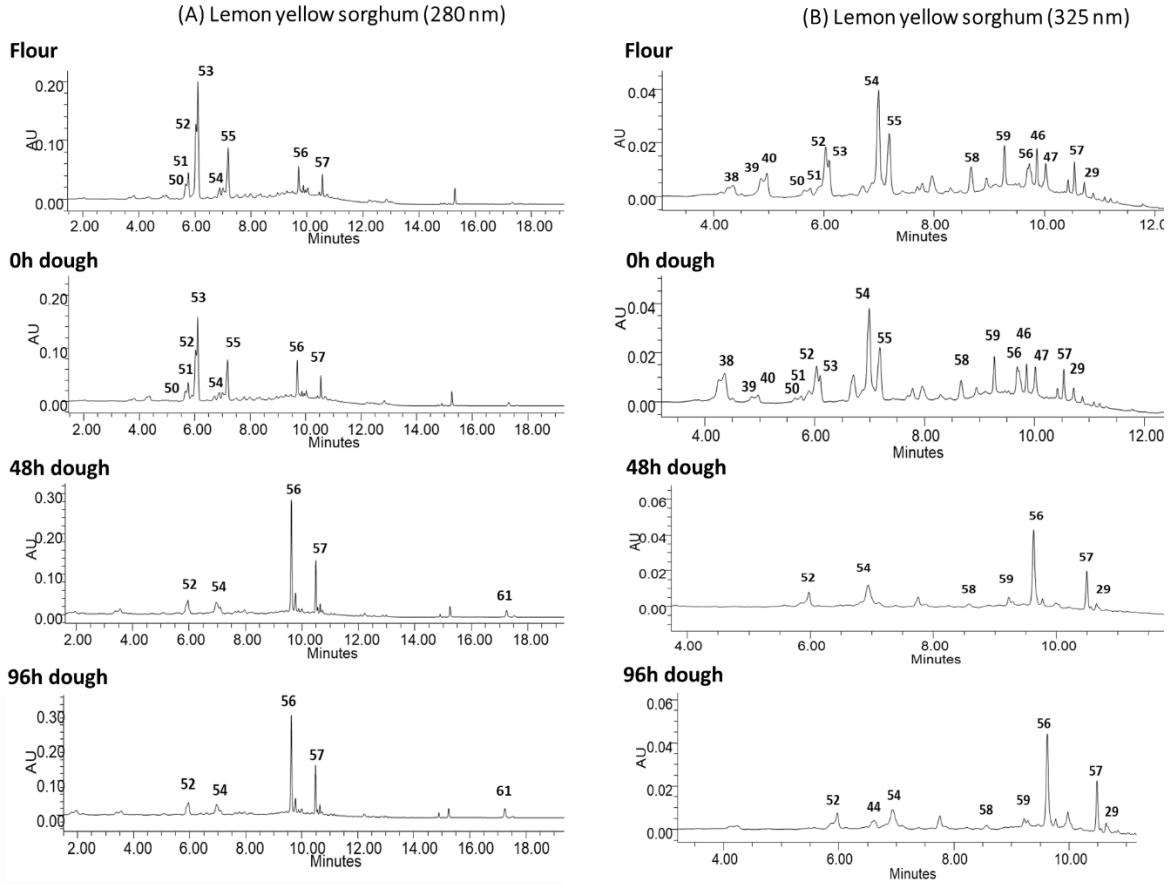

Figure 21: Effect of fermentation on UPLC profile of polyphenols identified in lemon yellow sorghum at 280 nm (A) at 325 nm (B). Non-fermented dough = 0 h and fermented doughs = 48 h and 96 h. Peak identities are as described in Section 4.3.2.1. Peak numbers 38-40, 44, 46, 47 correspond to peak numbers 2,3,4,8,9 in Table 3 and peak numbers 50-59 correspond to peak numbers 14-23 in Table 4. Other peak identities are as described in Section 6.3.2, Table 12.

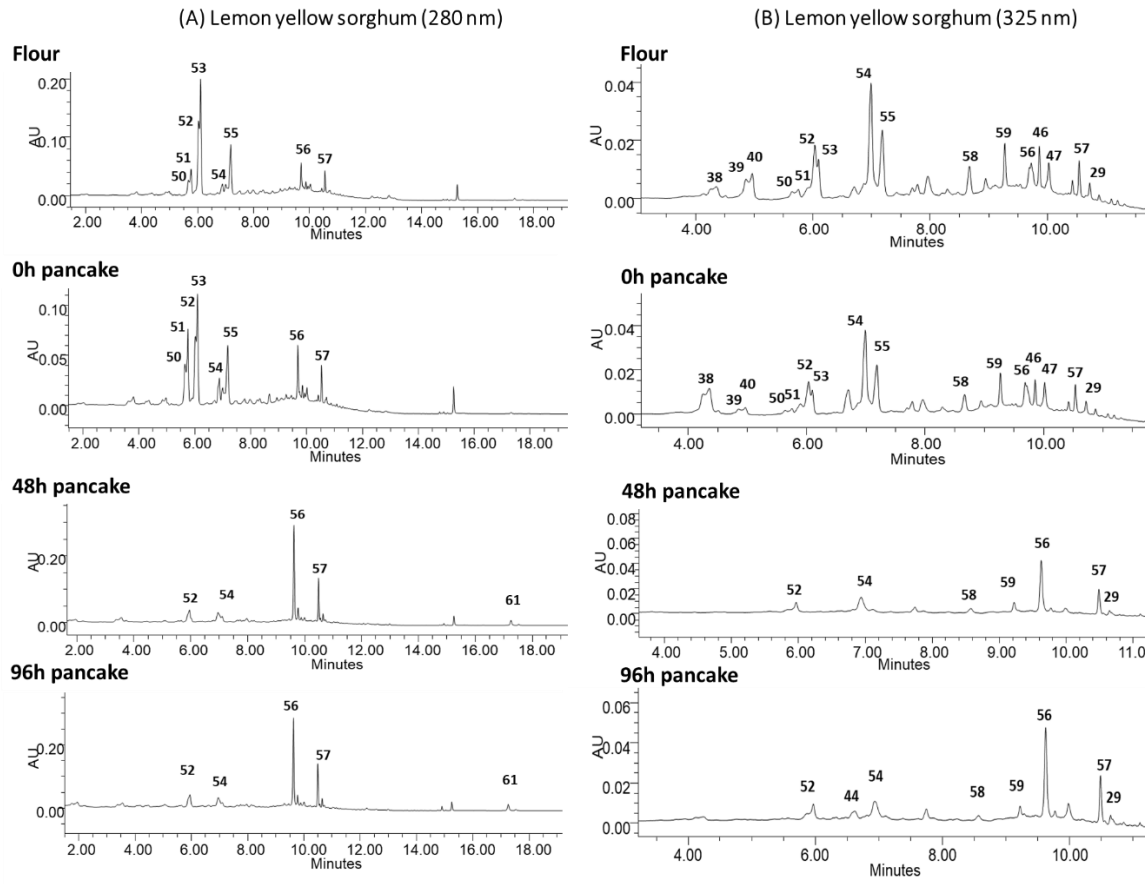

Figure 22: Effect of baking on UPLC profile of polyphenols identified in lemon yellow sorghum at 280 nm (A) and at 325 nm (B). Non-fermented pancakes = 0 h; fermented pancakes = 48 h and 96 h. Peak identities are as described in Section 4.3.2.1. Peak numbers 38-40, 44,46, 47 correspond to peak numbers 2,3,4,8,9 in Table 3 and peak numbers 50-59 correspond to peak numbers 14-23 in Table 4. Other peak identities are as described in Section 6.3.2, Table 12.

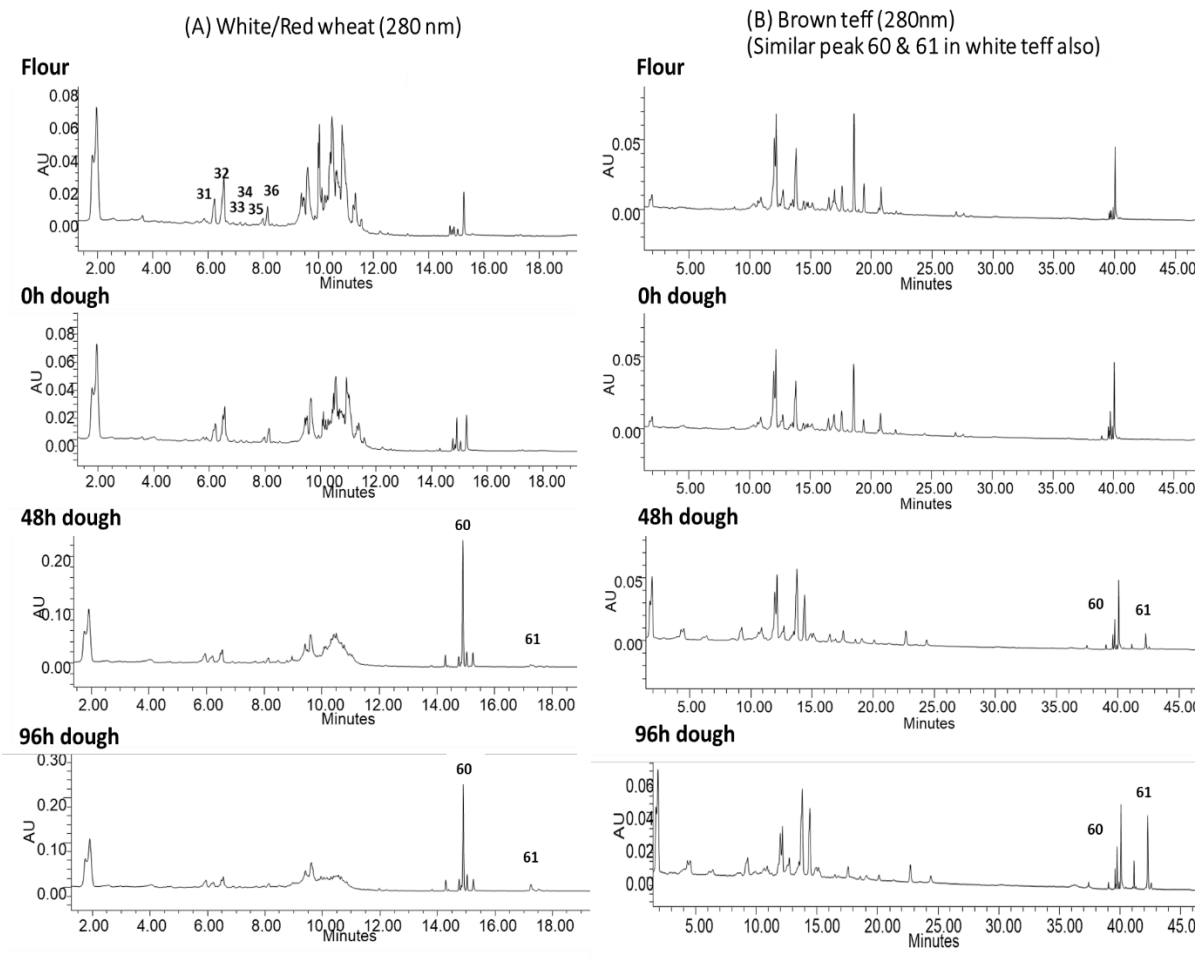

Figure 23 : Effect of fermentation on UPLC profile of polyphenols identified in white/red wheat at 280 nm (A) and white/brown teff at 280 nm (B). Non-fermented dough = 0 h and fermented doughs = 48 h and 96 h. Peaks 60 and 61 identities are as described in Section 6.3.2, Table 12. Peak 31- 36 (corresponding to peak numbers 1 – 6) identities are as described in Section 4.3.1.1 and Table 1.

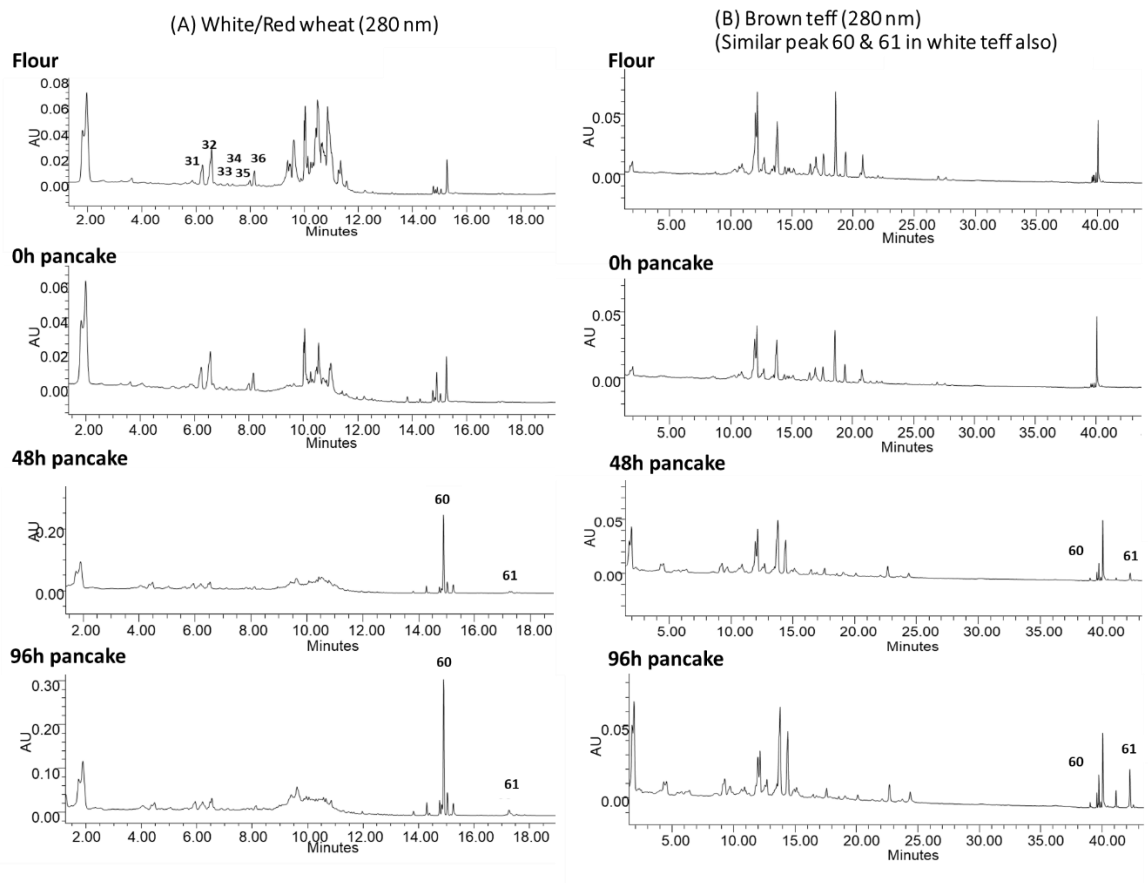

Figure 24: Effect of baking on UPLC profile of polyphenols identified in white/red wheat at 280 nm (A) and white/brown teff at 280 nm (B). Non-fermented pancake = 0 h; fermented pancakes = 48 h and 96 h. Peaks 60 and 61 identities are as described in Section 6.3.2, Table 12. Peak 31- 36 (corresponding to peak numbers 1 – 6) identities are as described in Section 4.3.1.1 and Table 1.

6.3.3. Effect of fermentation on profile and content of phenolic acids and flavonoids in wheat, sorghum and teff grains

The profile and content of phenolic acids and flavonoids were determined in wheat, sorghum and teff grains before (0 h) and after fermentation for 48 h and 96 h and after baking into a pancake in order to ascertain both structural and content changes. The outcomes are discussed with respect to specific flavonoid classes and phenolic acids identified across the different grains in order to understand if the effects on the flavonoid structure was similar irrespective of the grain type.

6.3.3.1. Effect of fermentation and baking on profile and content of flavonoid-O-glycosides

Peaks 9 and 22, 23 (Figs 25 & 26) were apigenin and luteolin O-glycosides in white and brown teff samples respectively, whereas peaks 50-55 and 58, 59 were flavanone-O-glycosides and their respective chalcones in lemon yellow sorghum (Figs 21 & 22; Table 13).

6.3.3.1.1. Effect of fermentation on profile and content of flavonoid-O-glycosides

The flavonoid-O-glycosides (peaks 9, 22, 23, 50-55 and 58, 59) showed significant reduction in fermented dough samples at 48 h and 96 h compared to non-fermented dough at 0 h in both white and brown teff and lemon-yellow sorghum samples (Table 13). Concurrently, there was significant increase in the respective aglycone forms of the compounds in the fermented doughs (48 h and 96 h) compared to dough at 0 h (peaks 29, 30 in teff samples and 56, 57 in lemon yellow sorghum samples). In lemon yellow sorghum, eriodictyol aglycone increased by 2.6 times in the fermented dough samples (48 h, 96 h) compared to dough at 0 h, and naringenin increased by 2.0 – 2.2 times in the fermented

dough samples (48 h, 96 h) compared to dough at 0 h, with the corresponding reduction in the O-glucoside forms (Table 13).

In the white teff non-fermented dough (0 h), there was trace levels of apigenin whereas in brown teff non-fermented dough (0 h) luteolin aglycone was not detected. However, in the white teff fermented dough samples (48 h and 96 h), apigenin aglycone content increased by 2.5 to 5.3 times in the 48 h and 96 h dough, respectively, compared to the non-fermented dough. Similarly, luteolin was detected in brown teff fermented dough samples (48 h and 96 h) (6.6 – 9.2 μg/g). This increase in flavone aglycones is likely be due to metabolism of apigenin-O-neohesperidoside (peak 9) and luteolin-O-glycosides (peak 22, 23), respectively, which showed a gradual reduction in content upon fermentation. These observations of cleavage of O-glucoside form to release corresponding aglycone was similar to those reported in a previous study evaluating the effect of fermentation on flavanone-O-glucosides in red sorghum [25].

6.3.3.1.2. Effect of baking on profile and content of flavonoid-O-glycosides

The content of all the flavonoid-O-glycosides (9, 22, 23, 50-55 and 58, 59) in non-fermented dough (0 h) compared to non-fermented pancake samples (0 h) were similar suggesting that the flavonoid-O-glycosides were stable to heat. In the fermented samples also (48 h and 96 h), comparison of dough to pancake, showed that the content of flavonoid-O-glucosides were similar (Table 13) further confirming their stability to heat treatment.

Table 13: Concentration (µg/g, dry weight basis) of flavonoid-O-glycosides and aglycones in non-fermented (0 h) and fermented (48 h and 96 h) white teff, brown teff and lemon-yellow sorghum dough and pancake samples[a.]

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
					Average µg/g of sample, db			
White Teff – Flavonoid-O-glycoside								
9	Apigenin-7-O-neohesperidoside[b]	105.8 ± 4.3 **A**	78.8 ± 2.0 **B**	79.4 ± 5.2 **B**	11.7 ± 1.7 **C**	17.1 ± 1.5 **C**	2.5 ± 0.2 **D**	8.0 ± 1.6 **C,D**
White Teff – Flavonoid aglycone								
29	Apigenin[d]	2.6 ± 0.8 **B**	6.4 ± 1.8 **B**	4.0 ± 0.8 **B**	16.1 ± 3.6 **A**	16.9 ± 3.6 **A**	21.5 ± 4.5 **A**	22.0 ± 4.6 **A**
Brown Teff – Flavonoid-O-glycosides								
22	Luteolin-7-O-glucoside[c]	78.2 ± 35.8	56.5 ± 3.9	50.8 ± 3.1	ND	ND	ND	ND
23	Luteolin-7-O-neohesperidoside[c]	108.8 ± 21.0	112.4 ± 5.9	97.6 ± 3.7	21.8 ± 1.6	22.9 ± 1.1	21.5± 1.4	24.9 ± 1.0
	Total	**187.1 ± 56.8 A**	**168.9 ± 9.8 A,B**	**148.4 ± 6.9 B**	**21.8 ± 1.6 C**	**22.9 ± 1.1 C**	**21.5± 1.4 C**	**24.9 ± 1.0 C**
Brown Teff – Flavonoid aglycone								
30	Luteolin[e]	ND	ND	ND	6.6 ± 2.8	8.6 ± 2.2	7.4 ± 1.5	9.2 ± 1.9
Lemon-Yellow sorghum – Flavonoid-O-glycosides								
50	Eriodictyol-7-O-galactoside[g]	68.7 ± 1.4	49.5 ± 3.4	95.2 ± 2.9	ND	ND	ND	ND
51	Eriodictyol-5-O-galactoside[g]	91.3 ± 0.9	66.9 ± 2.8	141.5 ± 2.6	ND	ND	ND	ND
52	Eriodictyol-7-O-glucoside[g]	280.9 ± 6.6	186.6 ± 16.1	177.9 ± 11.4	126.4 ± 1.2	143.2 ± 0.9	123.2 ± 4.5	199.9 ± 16.9
53	Eriodictyol-5-O-glucoside[g]	327.4 ± 13.8	250.2 ± 1.7	172.0 ± 11.6	ND	ND	ND	ND
54	Naringenin-7-O-galactoside[h]	46.6 ± 1.3	37.3 ± 2.2	56.4 ± 0.8	ND	ND	ND	ND
55	Naringenin-5-O-galactoside[h]	161.5 ± 5.1	153.9 ± 8.2	125.7 ± 9.0	115.8 ± 18.4	95.23 ± 16.80	122.61 ± 6.06	182.38 ± 5.37
58	Eriodictyol-7-O-galactoside chalcone[g]	29.6 ± 2.5	21.3 ± 7.6	80.5 ± 14.9	4.7 ± 1.8	12.1 ± 2.1	4.3 ± 0.9	14.2 ± 6.4
59	Naringenin-7-O-galactoside chalcone[h]	28.9 ± 7.5	19.0 ± 7.8	24.4 ± 7.2	5.6 ± 2.9	12.0 ± 2.4	3.5 ± 2.1	14.3 ± 2.7
	Total	**1034.9 ± 34.9 A**	**784.8 ± 46.8 B**	**873.6 ± 32.4 C**	**252.5 ± 16.3 E**	**262.5 ± 21.2 E**	**253.6 ± 11.0 E**	**410.8 ± 21.5 D**

Table 13 continued

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
					Average µg/g of sample, db			
Lemon-Yellow sorghum – Flavonoid aglycones								
56	Eriodictyol[g]	85.1 ± 12.1	93.2 ± 15.6	81.0 ± 12.5	239 ± 10	242 ± 13	252 ± 5	292 ± 16
57	Naringenin[h]	37.2 ± 4.8	36.2 ± 6.4	29.7 ± 4.5	70 ± 10	78 ± 5	73 ± 0	87 ± 6
	Total	**122.2 ± 16.9 C**	**129.5 ± 21.9 C**	**110.7 ± 16.8 C**	**309.1 ± 17.6 B**	**319.3 ± 18.3 B**	**324.7 ± 5.4 B**	**378.7 ± 21.5 A**

[a]Data are based on UPLC quantification of identified peaks and are expressed as mean ± standard deviation (n=3). [b]As apigenin-7-O-glucoside equivalents; [c]as luteolin-7-O-glucoside equivalents; [d] as apigenin equivalents; [e] as luteolin equivalents; [g] as eriodictyol equivalents; [h] as naringenin equivalents. Peaks that were not structurally identified are not included. Different capital letters indicate significant difference among all the samples in total content ($p<0.05$).

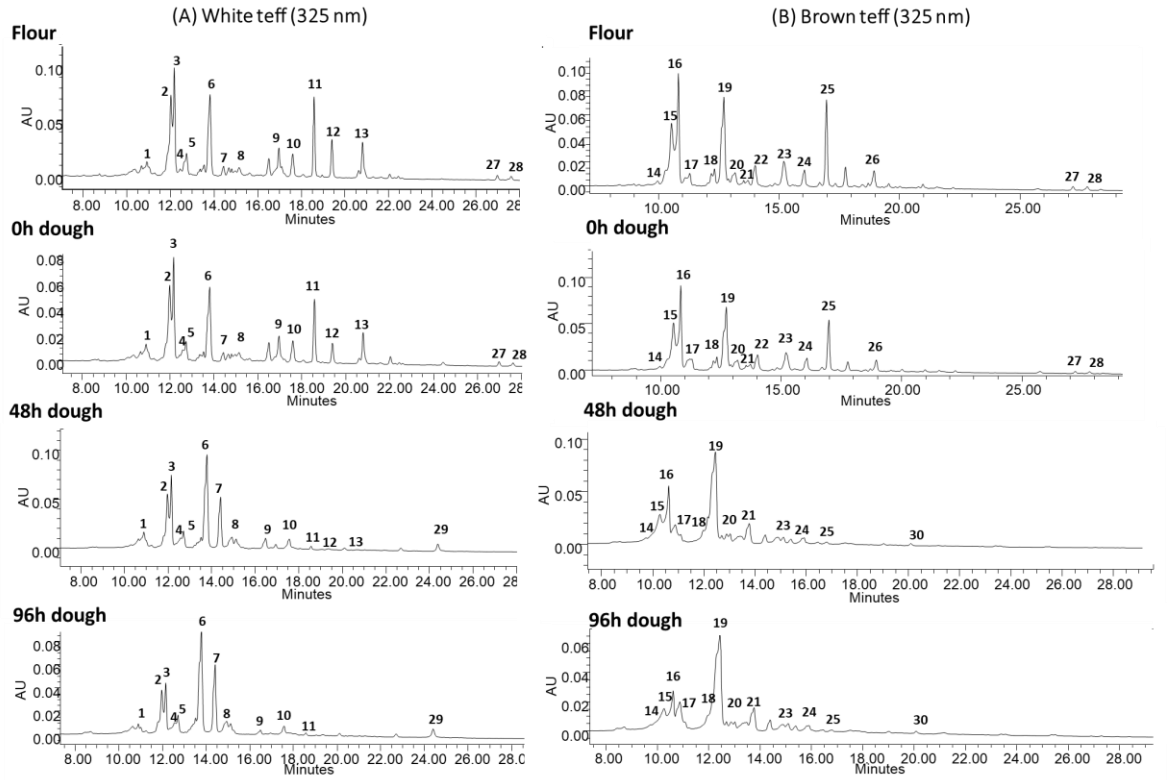

Figure 25: Effect of fermentation on UPLC profile of polyphenols identified in white teff (A) and brown teff (B) at 325 nm. Non-fermented dough = 0 h; fermented doughs = 48 h and 96 h. Peak identities (numbers 1-28) are as described in Section 5.3.1. and Table 9. Peaks 29, 30 corresponds to peak 12 and 13 as identified in Section 4.3.2.1, Table 4.

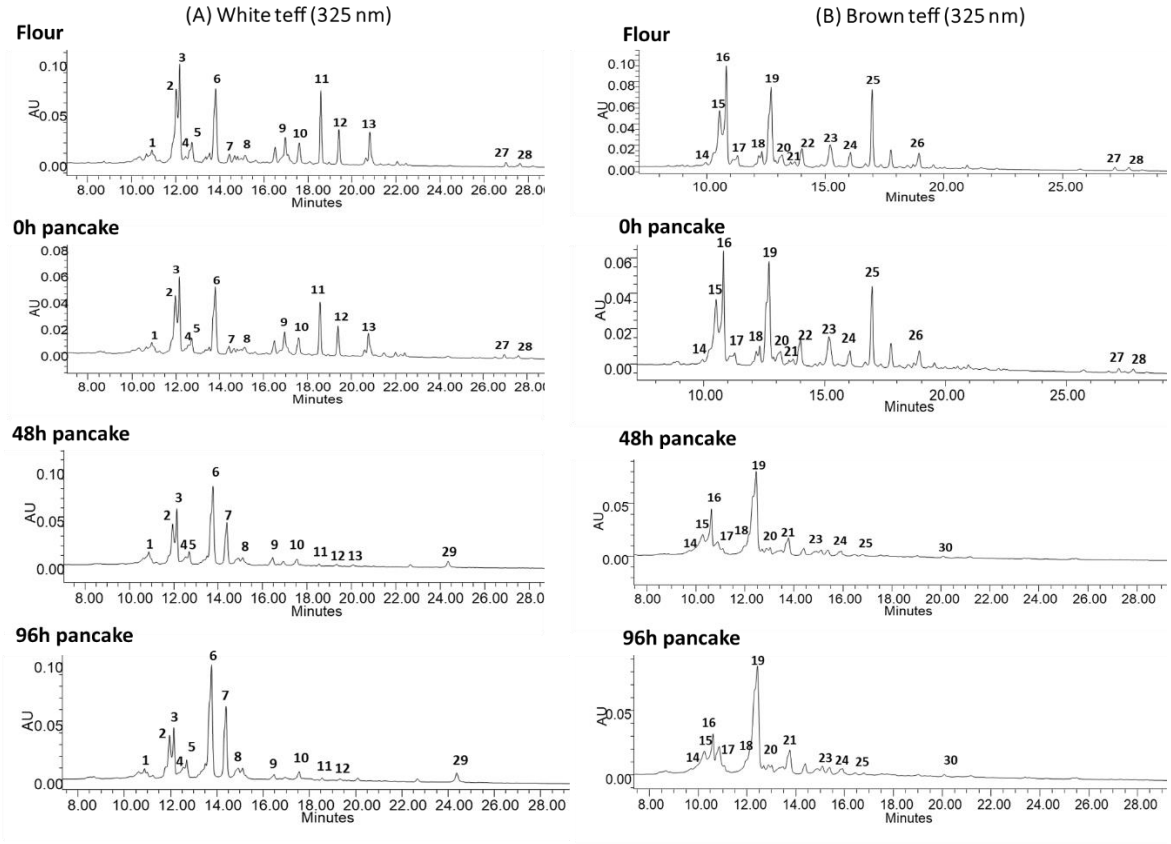

Figure 26: Effect of baking on UPLC profile of polyphenols identified in white teff (A) and brown teff (B) at 325 nm. Non-fermented pancake = 0 h; fermented pancakes = 48 h and 96 h. Peak identities (numbers 1-28) are as described in Section 5.3.1. and Table 9. Peaks 29, 30 corresponds to peak 12 and 13 as identified in Section 4.3.2.1, Table 4.

6.3.3.2. Effect of fermentation and baking on profile and content of flavonoid aglycones

Aglycone form of flavones - apigenin and luteolin (peak 29, 30) were present across white sorghum (Figs 19& 20), white teff and in fermented brown teff grains (Figs 25&26). Flavanone aglycones eriodictyol (peak 56) and naringenin (peak 57) was present in lemon-yellow sorghum grains (Figs 21& 22).

6.3.3.2.1. Effect of fermentation on profile and content of flavonoid aglycones

Effect of fermentation on aglycone form is discussed with respect to apigenin and luteolin in white sorghum grains (peak 29 and 30), as there are no other flavonoids forms in white sorghum, that could be converted to the aglycone form upon fermentation. As observed, fermentation of white sorghum dough for 48 h or 96 h did not show any changes in content of apigenin or luteolin compared to non-fermented dough (0 h) (Table 14; Figs 19&20). Thus, aglycone form of flavonoids are not metabolized when fermented. This is the first report in literature to observe the effect of natural fermentation on aglycone flavonoid forms.

6.3.3.2.2. Effect of baking on profile and content of flavonoid aglycones

The content of aglycone forms of apigenin (peak 29) and luteolin (peak 30) in white sorghum, apigenin (peak 29) in white teff and eriodictyol (peak 56) and naringenin (peak 57) in lemon yellow sorghum samples in the 0 h non-fermented dough compared to the 0 h non-fermented pancake samples were similar, indicating no effect of baking on the aglycone flavonoid forms in the different grains (Table 13&14). Even in the fermented products of white sorghum, white/brown teff and lemon-yellow sorghum, there was no change in aglycone flavonoid content when the fermented dough sample (48 h or 96 h) was baked into pancakes. Thus, the flavonoid aglycone forms were found to be stable to fermentation and subsequent heat treatment.

Table 14: Concentration (µg/g, dry weight basis) of flavone aglycones in white sorghum non-fermented (0 h) and fermented (48 h and 96 h) dough and pancake samples[a.]

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
				Average µg/g of sample, db				
White sorghum – Flavone aglycones								
30	Luteolin[e]	28.0 ± 6.0	25.6 ± 5.4	30.9 ± 5.9	27.3 ± 7.2	27.2 ± 7.3	25.3 ± 4.6	25.8 ± 5.2
29	Apigenin[d]	60.3 ± 10.2	62.5 ± 12.7	73.0 ± 12.7	62.1 ± 12.8	65.1 ± 16.4	54.9 ± 8.1	56.2 ± 8.8
	Total	**88.3 ± 16.2**	**88.1 ± 18.1**	**103.9 ± 18.6**	**89.4 ± 20.1**	**92.3 ± 23.7**	**80.2 ± 12.7**	**82.0 ± 14.0**

[a]Data are based on UPLC quantification of identified peaks and are expressed as mean ± standard deviation (n=3). [d] As apigenin equivalents. [e] As luteolin equivalents. Peaks that were not structurally identified are not included. Different letters indicate significant difference among all the samples in total content based on Tukey's HSD ($p<0.05$).

6.3.3.3. Effect of fermentation and baking on profile and content of flavone-di-C-glycosides

Flavone-di-C-glycosides were identified in white and red wheat samples (peaks 31-36) (Figs 27&28) as well as white and brown teff grains (peaks 1, 4, 5 and 14) (Figs 25&26).

6.3.3.3.1. Effect of fermentation on profile and content of flavone-di-C-glycosides

The content of apigenin-di-C-glycosides in the dough of both wheat and teff samples were generally similar across the different fermentation times (48 and 96 h) when compared to dough at 0 h (Table 15). This indicates that the process of fermentation employed in this study did not metabolize the di-C-linked glycosides. A previous study reported that vitexin and isovitexin (apigenin-8-C-glucoside and apigenin-6-C-glucoside, respectively), in mung bean milk did not show changes in structure or concentration upon fermentation with *Lactobacillus plantarum* WCFS1 strain, indicating that the mono-C-linked glycoside linkages are typically not metabolized when fermented with lactic acid bacteria [74]. This is the first study observing similar effects with flavone-di-C-glycosides.

6.3.3.3.2. Effect of baking on profile and content of flavone-di-C-glycosides

When wheat or teff dough samples were baked into pancakes, the levels of flavone-di-C-glycosides generally remained similar (at 0 h, 48 h and 96 h) (Table 15). This indicates that flavone-di-C-glycosides are stable under the baking conditions used in this study. To the best of our knowledge, this is the first report of the effect of fermentation and subsequent baking on flavone-di-C-glycosides.

Table 15: Concentration (μg/g, dry weight basis) of flavone-di-C-glycosides in white wheat, red wheat, white teff and brown teff non-fermented (0 h) and fermented (48 h and 96 h) dough and pancake samples[a.]

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
					Average μg/g of sample, db			
White wheat – Flavone-di-C-glycosides								
31	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	29.8 ± 1.1	24.1 ± 1.7	20.4 ± 1.2	22.8 ± 0.3	25.6 ± 4.9	23.8 ± 3.7	26.4 ± 0.6
32	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	52.2 ± 2.8	46.7 ± 2.4	35.5 ± 1.2	40.7 ± 0.4	35.8 ± 2.0	41.4 ± 3.7	45.3 ± 2.2
33	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	3.6 ± 0.3	2.3 ± 0.9	3.0 ± 1.3	3.1 ± 0.1	3.7 ± 0.2	6.7 ± 2.6	10.4 ± 0.4
34	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	3.6 ± 0.2	2.7 ± 1.1	2.6 ± 1.5	1.8 ± 0.1	5.7 ± 0.3	4.2 ± 2.4	13.9 ± 1.4
35	Apigenin-6-C-sinapoylpentoside-8-C-hexoside[b]	18.5 ± 0.4	10.1 ± 3.2	8.2 ± 2.8	5.5 ± 0.1	5.6 ± 0.5	14.5 ± 4.1	15.3 ± 0.2
36	Apigenin-8-C-sinapoylpentoside-8-C-hexoside[b]	33.1 ± 4.0	21.6 ± 4.3	19.5 ± 2.7	16.3 ± 0.4	12.8 ± 0.7	18.7 ± 2.4	24.2 ± 0.0
	Total	**140.8 ± 8.4 A**	**107.5 ± 13.3 B,C**	**89.3 ± 10.7 C**	**90.2 ± 0.8 B,C**	**89.2 ± 8.6 C**	**109.3 ± 18.8 B**	**135.5 ± 1.0 A**
Red wheat – Flavone-di-C-glycosides								
31	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	44.0 ± 2.4	36.2 ± 0.5	35.7 ± 0.5	32.8 ± 3.2	31.1 ± 0.5	33.4 ± 1.1	33.0 ± 2.1
32	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	78.0 ± 4.0	74.0 ± 0.6	60.6 ± 0.9	65.2 ± 3.1	56.0 ± 2.5	70.3 ± 3.1	70.3 ± 2.3
33	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	1.1 ± 0.2	0.6 ± 0.1	0.8 ± 0.2	4.1 ± 0.5	3.9 ± 0.2	5.9 ± 0.2	6.5 ± 0.3
34	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	2.2 ± 0.5	1.9 ± 0.0	2.4 ± 0.4	2.0 ± 0.1	3.1 ± 0.3	2.0 ± 0.3	2.8 ± 0.3

Table 15 continued

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
					Average µg/g of sample, db			
35	Apigenin-6-C-sinapoylpentoside-8-C-hexoside[b]	14.4 ± 1.7	11.1 ± 0.9	11.4 ± 0.7	8.2 ± 1.1	8.0 ± 1.3	7.4 ± 0.6	8.6 ± 0.4
36	Apigenin-8-C-sinapoylpentoside-8-C-hexoside[b]	37.9 ± 3.5	29.7 ± 0.5	32.1 ± 1.9	26.3 ± 0.8	23.9 ± 3.4	22.2 ± 2.0	26.1 ± 0.6
	Total	**177.59 ± 11.5 A**	**153.5 ± 0.9 B**	**143.0 ± 4.2 B,C**	**138.6 ± 6.7 C**	**125.9 ± 1.8 D**	**141.2 ± 6.2 C**	**147.3 ± 4.6 B,C**
White teff – Flavone-di-C-glycosides								
1	Apigenin-6,8-C-diglucoside[b]	69.7 ± 3.0	56.7 ± 1.1	37.2 ± 1.1	59.0 ± 3.6	51.8 ± 3.4	27.8 ± 6.4	28.8 ± 1.8
4	Apigenin-6-C-galactosyl-8-C-arabinoside[b]	30.1 ± 0.8	36.2 ± 1.1	31.3 ± 0.9	47.1 ± 3.1	44.2 ± 2.9	53.6 ± 2.3	51.0 ± 1.4
5	Apigenin-6-C-glucosyl-8-C-arabinoside[b]	44.3 ± 1.6	39.7 ± 0.1	31.0 ± 1.1	42.1 ± 3.3	35.1 ± 1.6	44.5 ± 2.5	39.5 ± 1.6
	Total	**144.1 ± 5.4 A,B**	**132.6 ± 2.3 A,B,C**	**99.4 ± 1.0 D**	**148.2 ± 9.9 A**	**131.2 ± 7.8 A,B,C**	**125.9 ± 10.1 B,C**	**119.3 ± 4.6 C**
Brown teff – Flavone-di-C-glycosides								
14	Luteolin-6,8-di-C-glucoside[c]	23.1 ± 2.2 **A**	24.2 ± 2.0 **A**	18.2 ± 1.2 **A**	15.2 ± 2.64 **A**	35.4 ± 6.1 **A**	0.5 ± 0.8 **A**	25.2 ± 21.8 **A**

[a]Data are based on UPLC quantification of identified peaks and are expressed as mean ± standard deviation (n=3). [b]As apigenin-7-O-glucoside equivalents. [c]As luteolin-7-O-glucoside equivalents. Peaks that were not structurally identified are not included. Different capital letters indicate significant difference among all the samples in total content based on Tukey's HSD ($p<0.05$).

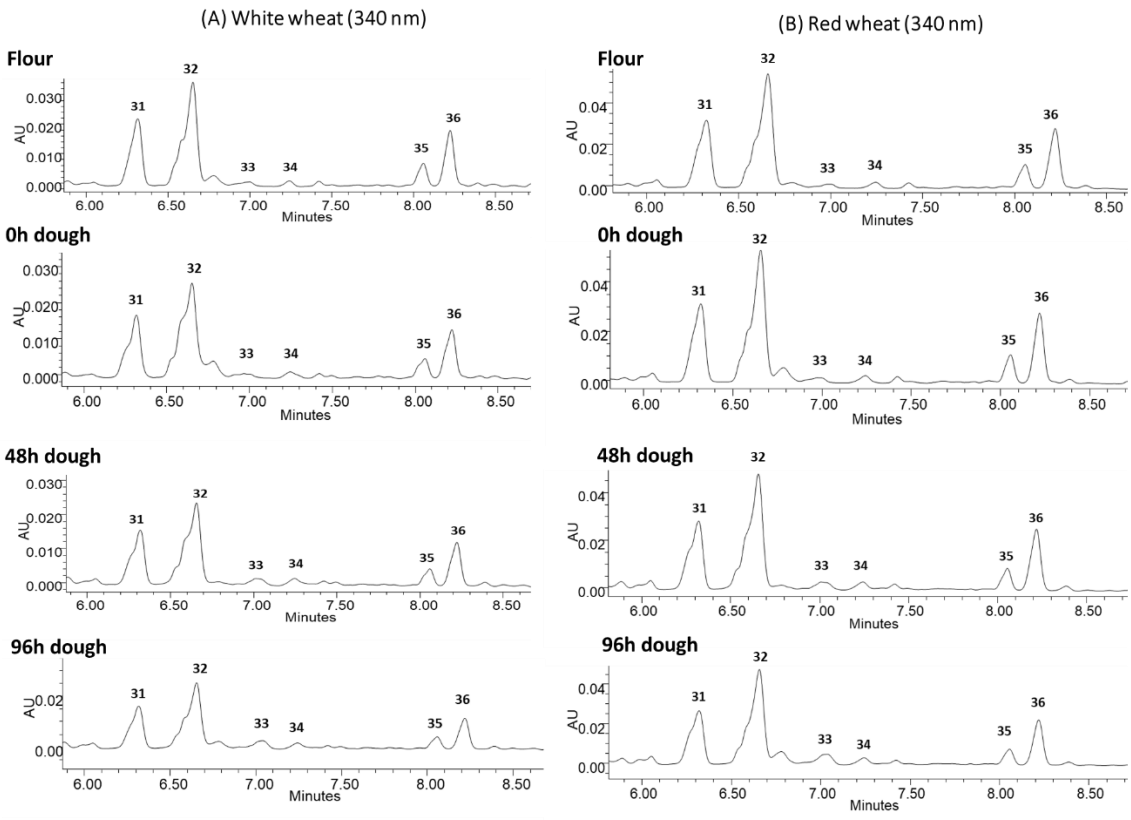

Figure 27: Effect of fermentation on UPLC profile of polyphenols identified in white wheat (A) and red wheat (B) at 340 nm. Non-fermented dough = 0 h and fermented doughs = 48 h and 96 h. Peak identities are as described in Section 4.3.1.1 and Table 1. Peaks 31-36 correspond to peak numbers 1-6 in Table 1.

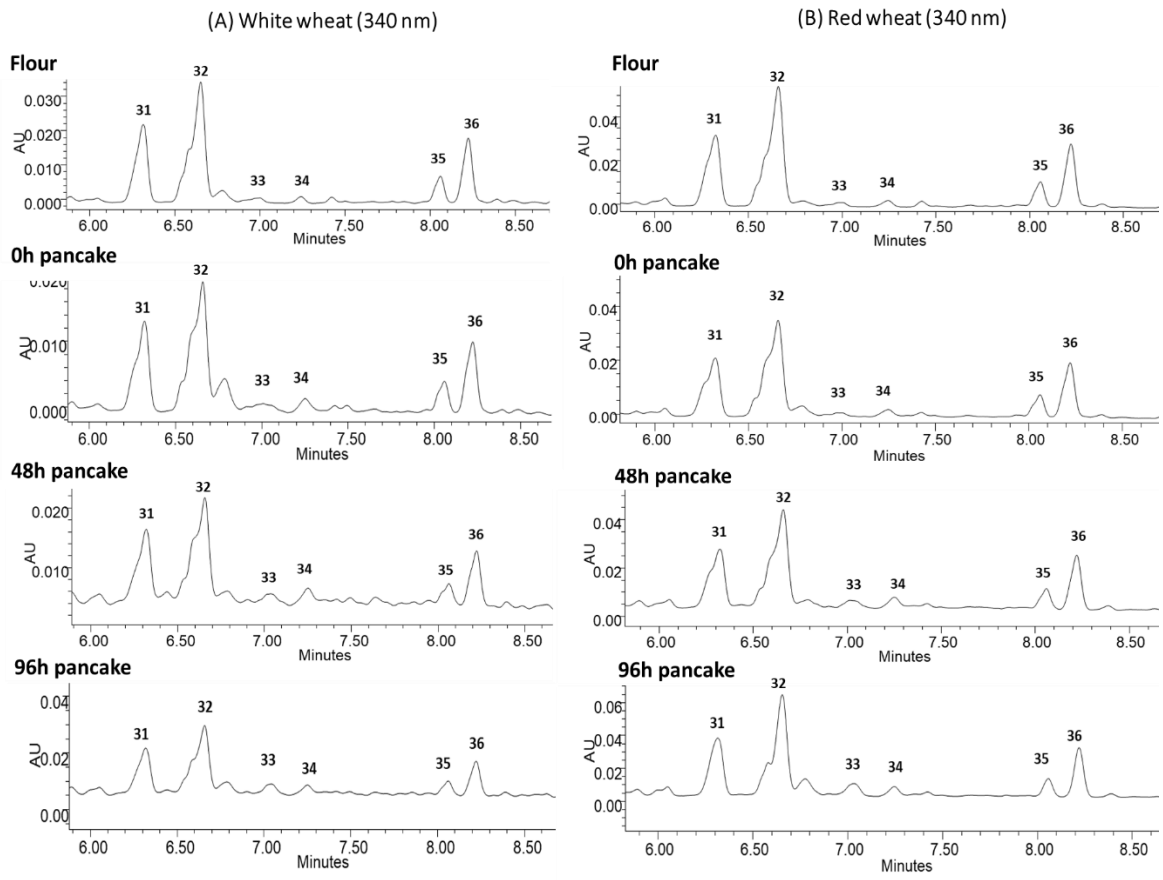

Figure 28: Effect of baking on UPLC profile of polyphenols in white wheat (A) and red wheat (B) at 340 nm. Non-fermented pancake = 0 h; fermented pancake = 48 h and 96 h. Peak identities are as described in Section 4.3.1.1 and Table 1. Peaks 31-36 correspond to peak numbers 1-6 in Table 1.

6.3.3.4. Effect of fermentation and baking on profile and content of flavone-O-glycoside-C-glycosides and acyl derivatives of flavone glycosides

Flavone-O-glycoside-C-glycosides (peaks 2, 3, 15, 16, 17) and acyl derivatives of flavone-O-glycoside-C-glycosides/flavone-mono-C-glycosides (peaks 10, 11, 12, 13, 24, 25 and 26) were all identified in white and brown teff grains (Figs 25&26).

6.3.3.4.1. Effect of fermentation on flavone-O-glycoside-C-glycosides and acyl derivatives of flavone glycosides

Flavone-O-glycoside-C-glycosides (peaks 2, 3, 15, 16, 17) showed significant reduction in content after 48 h and 96 h of fermentation compared to non-fermented dough at 0 h (Table 16). The decrease was gradual over the fermentation time. In general, the reduction among the peaks in 48 h dough compared to 0 h dough ranged between 10-35% and the reduction among the peaks in 96 h fermented dough compared to 0 h dough ranged between 31-55%. The observed reduction could be due to cleavage of O-glycoside linkage as this linkage is susceptible to microbial cleavage during fermentation [25]. In agreement with this observation, a corresponding increase in flavone-mono-C-glucoside was observed (peaks 6, 7 and 19) (Table 16). In general, across fermentation time, the mono-C-glycoside content in 48 h fermented dough increased by 1.6 – 7.4 times vs non-fermented dough (0 h). Whereas the mono-C-glycoside content in 96 h fermented dough increased by 1.8 – 19.4 times vs non-fermented dough (0 h)

Similarly, the acyl derivatives of flavone-O-glycoside-C-glycosides/flavone-mono-C-glycosides (peaks 10, 11, 12, 13, 24, 25 and 26) showed a significant reduction in content in fermented dough at 48 h and 96 h compared to dough at 0 h with no major differences when fermented for 48 or 96 h (Table 16). Peaks 10, 11, 24 and 25 were acylated to O-glucoside and hence the reduction could be either attributed to the cleavage of the flavonoid-O-glucoside bond or cleavage of acyl group from glycoside group. However, peaks 12, 13 and 26 were acylated on the C-glucoside pinpointing to the metabolism of the acyl-glucose linkage upon fermentation when C-linked (as mono-C-glycoside linkages to the flavonoid molecule are not metabolized when fermented [74]). These observed reductions in the acyl

derivatives could have also contributed to the observed increase in flavone-mono-C-glycoside contents (Table 16).

6.3.3.4.2. Effect of baking on flavone-O-glycoside-C-glycosides and acyl derivatives of flavone-C-glycosides

In general, the content of flavone-O-glycosides (peaks 2, 3, 15, 16, 17) as well as acyl derivatives of flavone-O-glycoside-C-glycosides/flavone-mono-C-glycosides (peaks 10, 11, 12, 13, 24, 25 and 26) in non-fermented pancake samples (0 h) were lower compared to non-fermented dough samples (0 h) (Table 16). However, in the 48 h and 96 h fermented pancake samples, the content was similar to that of 48 h or 96 h fermented dough. This could indicate a change in matrix composition upon fermentation causing differences in extractability of the compounds. In the process of fermentation, the action of endogenous and/or microbial enzymes as well as drop in pH, results in changes of macromolecules mainly forming the matrix of the product (e.g. starch degradation to fermentable sugars [177] or granule erosion [178], proteolysis [177] and/or unfolding of proteins resulting in weakening of structure [179] and solubilization of arabinoxylans [177]). These changes in matrix, could have led to increased extractability of the flavonoids in the fermented pancakes compared to the non-fermented pancakes.

The content of mono-C-glycosides (peaks 6,7,19) produced upon fermentation of the flavone-O-glycoside-C-glycosides and acyl derivatives in pancake samples at 0 h, 48 h and 96 h, were generally similar to the corresponding dough samples, indicating their stability to baking.

Table 16: Concentration (μg/g, dry weight basis) of flavone-O-glycoside-C-glycosides and acyl derivatives of flavone-O-glycoside-C-glycoside and flavone-mono-C-glycosides in white wheat, red wheat, white teff and brown teff non-fermented (0 h) and fermented (48 h and 96 h) dough and pancake samples[a.]

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
					Average μg/g of sample, db			
White teff – Flavone-O-glycoside-C-glycoside								
2	Apigenin-8-C-glucosyl-7-O-glucoside[b]	263.5 ± 8.4	215.1 ± 2.2	171.5 ± 2.4	189.7 ± 5.7	160.0 ± 4.3	148.7 ± 5.1	129.8 ± 2.6
3	Apigenin-6-C-glucosyl-2"-O-glucoside[b]	207.1 ± 9.6	166.3 ± 2.2	129.3 ± 2.1	148.6 ± 7.0	129.9 ± 4.6	99.9 ± 2.3	91.5 ± 1.1
	Total	**470.6 ± 18.0 A**	**381.3 ± 4.28 B**	**300.8 ± 4.4 D**	**338.3 ± 12.7 C**	**289.9 ± 8.3 D**	**248.5 ± 7.5 E**	**221.4 ± 3.6 F**
Brown teff – Flavone-O-glycoside-C-glycoside								
15	Luteolin -8-C-glucosyl-7-O-glucoside isomer[c]	356.0 ± 45.1	343.7 ± 12.5	257.4 ±4.4	224.9 ± 21.4	194.1 ± 13.8	172.4 ± 12.3	178.4 ± 12.4
16	Luteolin -6-C-glucosyl-7-O-glucoside isomer[c]	342.7 ± 5.1	329.9 ± 11.1	234.9 ± 1.3	232.3 ± 20.2	207.9 ± 5.8	148.0 ± 6.6	153.8 ± 5.1
17	Luteolin-8-C-glucosyl-7-O-neohesperidoside[c]	56.4 ± 6.4	112.8 ± 7.1	44.2 ± 2.7	64.0 ± 17.2	75.1 ± 4.8	122.3 ± 7.7	104.5 ± 6.4
	Total	**721.9 ± 48.4 A**	**697.8 ± 25.6 A**	**510.5 ± 4.1 B**	**472.4 ± 67.7 B**	**437.4 ± 12.9 B,C**	**320.7 ± 18.8 C,D**	**357.3 ± 15.9 D**

Table 16 continued

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
					Average µg/g of sample, db			
White teff – Acyl derivatives								
10	Apigenin-7-O-(6”-syringyl)glucosyl-6-C-glucoside[b]	81.6 ± 3.6	66.8 ± 1.7	57.9 ± 3.3	52.9 ± 4.9	36.6 ± 2.0	28.9 ± 1.4	25.9 ± 2.8
11	Apigenin-7-O-(6”-syringyl)arabinosyl-8-C-glucoside[b]	183.3 ± 8.5	128.0 ± 2.6	118.0 ± 3.7	6.4 ± 0.3	4.8 ± 0.4	4.3 ± 0.3	4.3 ± 0.3
12	Apigenin-8-C-(6”-acetyl) glucoside[b]	60.9 ± 2.7	26.4 ± 0.9	42.5 ± 1.7	1.8 ± 0.1	4.7 ± 0.6	ND	2.6 ± 0.1
13	7-O-methyl apigenin-8-C-(2”-succinyl) arabinoside[b]	71.8 ± 3.7	49.7 ± 0.9	47.8 ± 2.1	0.5 ± 0.6	0.4 ± 0.1	ND	ND
	Total	**397.6 ± 18.4 A**	**270.8 ± 5.5** B	**266.3 ± 10.7** B	**61.6 ± 5.6 C**	**46.3 ± 2.9 C,D**	**34.4 ± 1.7 D**	**32.7 ± 3.0 D**
Brown teff – Acyl derivatives								
24	Luteolin-7-O-(6”-syringly)glucosyl-6-C-glucoside[c]	152.5 ± 115.7	88.5 ± 9.9	60.8 ± 6.6	49.0 ± 4.2	45.8 ± 2.9	44.9 ± 2.6	43.9 ± 1.5
25	Luteolin-7-O-(2”-syringyl)arabinosyl-6-C-glucoside[c]	210.4 ± 136.7	226.2 ± 9.1	192.2 ± 3.3	11.7 ± 2.5	14.3 ± 1.3	7.5 ± 0.3	7.0 ± 0.3
26	Luteolin-8-C-(6”-diacetyl)glucoside[c]	54.2 ± 3.5	45.7 ± 2.8					
	Total	**471.7 ± 42.7 A**	**427.1 ± 24.6 A**	**350.6 ± 13.4 B**	**82.4 ± 4.5 C**	**83.1 ± 5.3 C**	**73.9 ± 4.3 C**	**75.8 ± 2.7 C**
White teff – Flavone-mono-C-glycosides								
6	Apigenin-8-C-glucoside[b]	181.4 ± 11.6	141.3 ± 3.9	133.7 ± 4.0	231.5 ± 10.1	227.3 ± 9.4	254.9 ± 8.0	253.9 ± 4.7
7	Apigenin-6-C-glucoside[b]	19.3 ± 0.7	14.7 ± 0.4	13.1 ± 1.1	111.3 ± 4.7	107.5 ± 4.3	144.8 ± 3.6	137.1 ± 1.7
	Total	**200.6 ± 12.2 C**	**155.9 ± 4.2 D**	**146.8 ± 5.0 D**	**342.7 ± 14.7 B**	**334.8 ± 13.7 B**	**399.7 ± 11.5 A**	**391.0 ± 6.5 A**

Table 16 continued

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
				Average µg/g of sample, db				
Brown teff – Flavone-mono-C-glycoside								
19	Luteolin-8-C-glucoside[c]	299.8 ± 13.3 **B**	274.2 ± 9.4 **B,C**	233.8 ± 3.6 **C**	516.5 ± 26.9 **A**	504.2 ± 26.4 **A**	512.4 ± 35.8 **A**	539.5 ± 16.7 **A**

[a]Data are based on UPLC quantification of identified peaks (Table 1) and are expressed as mean ± standard deviation (n=3). [b]As apigenin-7-O-glucoside equivalents. [c]As luteolin-7-O-glucoside equivalents. ND: not detected. Peaks that were not structurally identified are not included. Different capital letters indicate significant difference among all the samples in total content based on Tukey's HSD ($p<0.05$).

To the best of our knowledge, this is the first report of the effect of fermentation and baking on flavone-O-C-glycosides, mono-C-glycosides and their acyl derivatives. A previous study showed the reduction in content of malonyl and acetyl daidzein and genistein respectively in yellow soybean flour when fermented [180].

6.3.3.5. Effect of fermentation and baking on profile and content of phenolic acids

Peaks 27, 28 and 37-49 were phenolic acid/phenolic acid esters of glycerol and spermidine found in white sorghum (Figs 19&20) , lemon yellow sorghum (Figs 21&22) and teff samples (Figs 25&26).

6.3.3.5.1. Effect of fermentation on profile and content of phenolic acids

White sorghum had high abundance of phenolic acids compared to lemon yellow and teff samples and served as a better model to study phenolic acid metabolism. Results showed that both phenolic acid ester derivatives of glycerol as well as spermidine significantly reduced in the fermented dough samples (48 h and 96 h) compared to non-fermented dough (0 h) (Table 17). Correspondingly, there was an increase in phenolic acid metabolites-dihydroferulic acid (metabolite of ferulic acid) and vinyl catechol (metabolite of caffeic acid) which are common food phenolic acid metabolites [25, 173] (Table 17). These phenolic acid metabolites were only detected in the 48 h and 96 h fermented dough samples. Also, there was significant increase in ferulic acid in the fermented dough at 48 h and 96 h (2.6 to 2.8 times the content of non-fermented dough (0 h)), with no significant difference between the fermentation times (Table 17). Ferulic acid is abundant in the bound form in sorghum [25] and the increase could likely be due to esterase activity of lactic acid bacteria breaking ester linkages to release the bound phenolic acid [68].

Since lemon yellow and teff samples had lower initial content of phenolic acid glycerol ester derivatives, the acids were completely metabolized when fermented and not detected in 48 h and 96 h fermented dough samples (Table 17). No corresponding metabolite could be detected in these samples likely due to their low abundance. However, ferulic acid was detected in the 96 h lemon yellow fermented dough, likely being released from the bound form (Fig 21 and table 17).

Other phenolic acids released from the bound form in wheat, teff and sorghum samples were the methyl gallate derivatives (as explained for peaks 60 and 61, section 6.3.2). There was a general trend for the methylgallate derivatives increasing in abundance with fermentation time (48 h to 96 h) and the maximum abundance was observed in wheat samples (Table 17). They were not detected in the 0 h dough samples (Figs 19-24).

6.3.3.5.2. Effect of baking on profile and content of phenolic acids

In general, the content of phenolic acids in pancake samples at different times (0 h, 48 h, 96 h) was either similar or higher to the corresponding dough samples at the same time points. This difference could be attributed to changes in matrix during fermentation increasing the extraction of phenolic acids in fermented pancake samples (Table 17).

Table 17: Concentration (µg/g, dry weight basis) of phenolic acids in white wheat, red wheat, white teff, brown teff, white sorghum and lemon yellow sorghum non-fermented (0 h) and fermented (48 h and 96 h) dough and pancake samples[a.]

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
		Average µg/g of sample, db						
Phenolic acids								
White sorghum								
37	2-O-caffeoylglycerol-O-glucoside [f]	32.3 ± 1.0	38.9 ± 1.0	40.9 ± 0.0	8.9 ± 2.4	14.0 ± 0.2	ND	ND
38	1-O-caffeoylglycerol-O-glucoside [f]	38.2 ± 0.1	48.3 ± 0.4	52.4 ± 1.1	10.8 ± 1.1	9.4 ± 0.0	11.3 ± 0.2	14.3 ± 0.3
39	2-O-caffeoylglycerol [f]	25.2 ± 1.2	16.4 ± 0.4	22.6 ± 0.2	5.6 ± 1.3	7.4 ± 0.3	ND	ND
40	1-O-caffeoylglycerol [f]	31.0 ± 1.7	21.9 ± 0.7	28.9 ± 0.7	4.1 ± 1.1	2.5 ± 0.1	ND	ND
41	N, N' - Dicaffeoylspermidine [f]	31.1 ± 0.8	38.5 ± 0.9	39.9 ± 1.0	13.0 ± 5.1	19.2 ± 0.7	10.0 ± 0.5	8.4 ± 1.1
42	N, N' - Dicaffeoylspermidine [f]	29.4 ± 1.9	21.6 ± 1.3	26.2 ± 3.9	ND	ND	ND	ND
43	Dihydroferulic acid[i]	ND	ND	ND	6.8 ± 0.0	9.7 ± 0.5	4.7 ± 0.1	4.8 ± 3.7
44	Ferulic acid[i]	21.2 ± 1.1	27.7 ± 0.5	28.8 ± 1.6	73.6 ± 4.4	88.8 ± 3.4	79.9 ± 3.2	72.3 ± 2.1
45	Vinyl catechol	ND	ND	ND	D	D	D	D
46	Dicaffeoylglycerol [f]	45.5 ± 4.3	40.0 ± 2.8	50.4 ± 1.1	13.4 ± 1.5	28.3 ± 2.0	ND	ND
47	Dicaffeoylglycerol [f]	137.3 ± 10.4	121.5 ± 9.3	139.0 ± 4.1	27.2 ± 3.7	17.4 ± 2.4	ND	ND
48	p-coumaroyl-caffeoyl-glycerol isomer[j]	140.6 ±20.9	133.5 ± 3.5	156.3 ± 2.5	33.3 ± 6.4	74.6 ± 5.4	ND	ND
49	Feruloyl-caffeoyl-glycerol[i]	85.3 ± 3.0	78.2 ± 11.5	83.6 ± 4.2	6.9 ± 2.3	6.3 ± 8.8	ND	ND
61	Methyl gallate derivative [k]	ND	ND	ND	157.2 ± 5.7	110.5 ± 1.6	146.0 ± 2.1	99.4 ± 2.8
	Total	**617.0 ± 38.3 B**	**586.6 ± 22.6 B**	**669.2 ± 15.4 A**	**358.6 ± 7.7 C**	**277.4 ± 4.6 C**	**251.8 ± 5.0 D**	**199.2 ± 6.4 E**

Table 17 continued

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
					Average µg/g of sample, db			
Lemon yellow sorghum								
38	1-O-caffeoylglycerol-O-glucoside [f]	17.3 ± 0.5	29.2 ± 1.8	21.6 ± 2.2	ND	ND	ND	ND
39	2-O-caffeoylglycerol [f]	10.1 ± 0.7	0.3 ± 0	10.1 ± 0.5	ND	ND	ND	ND
40	1-O-caffeoylglycerol [f]	10.8 ± 0.9	0.7 ± 0.1	9.3 ± 0.2	ND	ND	ND	ND
46	Dicaffeoylglycerol [f]	36.0 ± 7.5	40.9 ± 26.1	26.5 ± 4.0	ND	ND	ND	ND
47	Dicaffeoylglycerol [f]	56.5 ± 10.1	42.8 ± 12.1	39.9 ± 7.1	ND	ND	ND	ND
44	Ferulic acid[i]	ND	ND	ND	ND	ND	9.8 ± 1.5	17.4 ± 0.8
61	Methyl gallate derivative [k]	ND	ND	ND	47.1 ± 0.9	42.9 ± 0.2	66.4 ± 1.1	54.3 ± 0.2
	Total	**130.9 ± 18.2 A**	**113.8 ± 37.4 A**	**107.4 ± 11.5 A**	**47.1 ± 0.9 C**	**42.9 ± 0.2 C**	**76.3 ± 2.5 B**	**73.0 ± 1.1 B**
White teff								
27	Dicoumaroylglycerol[j]	13.8 ± 2.5	8.9 ± 0.6	8.5 ± 0.6	ND	ND	ND	ND
28	p-coumaroyl-feruloyl-glcerol[j]	15.0 ± 2.2	8.7 ± 0.7	8.5 ± 0.4	ND	ND	ND	ND
60	Methyl gallate derivative [k]	ND	ND	ND	32.4 ± 0.8	26.8 ± 0.6	37.3 ± 0.4	31.1 ± 1.0
61	Methyl gallate derivative [k]	ND	ND	ND	33.7 ± 1.2	25.8 ± 1.2	102.5 ± 1.4	58.4 ± 1.9
	Total	**28.8 ± 4.7 E**	**17.6 ± 1.3 F**	**16.9 ± 1.1 F**	**66.1 ± 1.9 C**	**52.6 ± 1.8 D**	**139.7 ± 1.8 A**	**89.5 ± 2.9 B**
Brown teff								
27	Dicoumaroylglycerol[j]	7.6 ± 0.5	6.2 ± 1.1	6.5 ± 0.4	ND	ND	ND	ND
28	p-coumaroyl-feruloyl-glcerol[j]	10.9 ± 0.6	7.1 ± 0.8	7.1 ± 0.4	ND	ND	ND	ND
60	Methyl gallate derivative [k]	ND	ND	ND	135.2 ± 8.0	106.0 ± 4.6	137.3 ± 7.4	118.7 ± 5.5
61	Methyl gallate derivative [k]	ND	ND	ND	42.4 ± 0.7	31.7 ± 1.7	82.7 ± 4.8	57.6 ± 2.2
	Total	**18.5 ± 1.1 D**	**13.3 ± 1.9 D**	**13.6 ± 0.7 D**	**177.6 ± 8.7 B**	**137.7 ± 6.3 C**	**220.0 ± 12.2 A**	**176.3 ± 7.7 B**

Table 17 continued

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
					Average µg/g of sample, db			
White wheat								
60	Methyl gallate derivative [k]	ND	ND	ND	154.4 ± 2.6	146.3 ± 7.9	90.0 ± 6.3	122.3 ± 0.7
61	Methyl gallate derivative [k]	ND	ND	ND	17.3 ± 1.7	20.6 ± 3.6	40.5 ± 2.4	31.3 ± 0.2
	Total				**171.6 ± 4.3 A**	**166.9 ± 4.3 A**	**130.5 ± 6.5 C**	**153.6 ± 0.6 B**
Red wheat								
60	Methyl gallate derivative [k]	ND	ND	ND	194.1 ± 6.1	202.8 ± 9.9	231.7 ± 8.7	233.0 ± 5.9
61	Methyl gallate derivative [k]	ND	ND	ND	12.5 ± 0.5		29.9 ± 1.5	21.5 ± 0.9
	Total				**206.6 ± 5.6 B**	**202.8 ± 9.9 B**	**261.6 ± 10.2 A**	**254.6 ± 6.8 A**

[a]Data are based on UPLC quantification of identified peaks and are expressed as mean ± standard deviation (n=3). [f] As caffeic acid equivalents. [i] As ferulic acid equivalents.[j] as coumaric acid equivalents. [k] as gallic acid equivalents. ND: not detected. D: detected but not quantified. Peaks that were not structurally identified are not included. Different capital letters indicate significant difference among all the samples in total content based on Tukey's HSD ($p<0.05$).

6.3.3.6. Summary on effect of fermentation and baking on different polyphenol forms

In general, it can be inferred that flavone-di-C-glycosides, flavone-mono-C-glycosides and aglycone forms of flavonoids are not metabolized when fermented by lactic acid bacteria. Flavone-O-glycoside-C-glycoside and acyl derivatives of flavone-O-glycoside-C-glycoside/flavone-mono-C-glucosides are metabolized when fermented to their respective mono-C-glucoside forms. Flavonoid-O-glucosides are metabolized to respective aglycone forms, while phenolic acid esters in cereal grains are transformed or metabolized to mainly dihydroferulic acid and vinyl catechol. Certain bound phenolic acids, ferulic acid and methylgallate derivatives can be released in the process of fermentation.

Baking did not significantly alter the profile and content of flavonoids. Previous studies have indicated that flavonoids and phenolic acids were generally stable when heated to 100-125 °C for around 20-30 min [181-182]. This study confirms the thermal stability of the flavonoids and phenolic acids in fermented and non-fermented cereal grains in a food matrix.

The increase in level of aglycones upon fermentation (due to cleavage of O-linked flavonoid-glycosides) and the corresponding thermal stability of the aglycone forms could be an important advantage in terms of their bioavailability. O-linked glycosides are typically hydrolyzed to aglycones in the small intestine for absorption (with enzyme lactase phlorizin hydrolase, LPH) [17] and the rate of hydrolysis could vary from individual to individual and could be affected by factors such as illness, diet or age [180]. Thus, the process of fermentation may be useful in providing the health beneficial aglycones and increasing their bioavailability.

On the other hand, certain studies have shown that flavone-mono-C-glycosides are present intact in plasma and urine of humans [97]. Flavone-C-multiglycosides (especially flavone-O-glycoside-C-glycoside forms) can also be absorbed intact through the small intestine and distributed to various tissues [6]. Thus, microbial deglycosylation during fermentation may not be a pre-requisite for flavonoid-C-glycoside absorption from the small intestine [6, 94]. Thus, stability of the C-glycosides during fermentation could be beneficial owing to their health benefits [6].

The metabolism of phenolic acids into their corresponding small molecule metabolites improves their absorption in the small intestine [141]. And lastly, the process of fermentation saw the release of bound polyphenols from the grains thus increasing their bioaccessibility. Therefore, fermentation could be employed as a strategic tool to improve the bioavailability and hence potential bioactivity of polyphenols from whole grains.

6.3.4. Effect of fermentation and baking on extractable phenol content and antioxidant activity of wheat, sorghum and teff fermented dough and pancakes

In order to understand if the observed structural and content changes in the process of fermentation could have an impact on their bioactivity, we tested the antioxidant activity of the fermented dough and pancake samples. Though previous studies have indicated the increase in antioxidant capacity of grain flours when fermented [68, 74, 183], there is lack of association between structural changes observed with respect to specific classes of flavonoids and phenolic acids and corresponding antioxidant capacity.

6.3.4.1. Effect of fermentation and baking on extractable phenol content (EPC) of wheat, sorghum and teff fermented dough and pancakes

The extractable phenol content of the different samples are presented in Table 18.

6.3.4.1.1. Effect of fermentation on EPC of wheat, teff and sorghum fermented grains

Across the different grains, the increase in EPC of fermented dough samples at 48 h ranged from 1.2 – 1.7 times of non-fermented dough at 0 h, and at 96 h, ranged from 1.4 – 2.1 times of the dough at 0 h. Thus, there was a gradual increase in EPC with fermentation time. The highest increase in EPC was observed in fermented wheat dough samples compared to their corresponding non-fermented samples (approx. 2 times increase in fermented dough at 96 h compared to 0 h non-fermented dough), whereas the increase in EPC in teff and sorghum fermented samples was within a similar range (approx. 1.5 times increase in fermented dough at 96 h compared to their non-fermented 0 h counterparts) (Table 18).

6.3.4.1.2. Effect of baking on EPC of wheat, teff and sorghum fermented grains

In fermented pancake samples, at 48 h there was a 1.5 – 2.7 times increase in EPC compared to non-fermented pancake at 0 h; at 96 h there was a 1.7 – 3 times increase in EPC compared to pancake at 0h, among the different grains (Table 18). The highest increase in EPC was observed in fermented wheat pancake samples compared to their corresponding non-fermented samples (approx. 3 times increase in fermented pancake samples at 96 h compared to 0 h non-fermented pancake), whereas the increase in EPC in teff and sorghum fermented samples was within a similar range (approx. 1.7-2.2 times increase in fermented pancake samples at 96 h compared to their non-fermented 0 h counterparts) (Table 18). At each time point of fermentation (48 h and 96 h), there was a significant increase in the phenol content when baked into a pancake compared to the corresponding dough sample, except for the teff samples wherein phenol contents were similar.

Thus, there were differences in EPC changes across the different grains, and EPC increased with increase in fermentation time across all the grains. Several enzymes involved in the process of fermentation such as esterases, xylanases, amylases, proteases and phenoloxidases could have led to release of bound phenolics and caused changes in the matrix of grains that could lead to increased extractability of the phenols in the fermented samples [68, 184]. As previously noted, ferulic acid in sorghum and the methyl gallate ester derivatives (peaks 60 and 61) (Table 12; Fig 18) in wheat, teff, and sorghum increased in the fermented samples, likely being released from their bound forms. The content of methyl gallate derivatives in wheat samples was greater than teff which was greater than sorghum (Table 17). Furthermore, the matrix changes could be different among the grains as they

vary in their macromolecular composition. All this could have contributed to the variable increase in EPC in the fermented samples in different grains, with the highest increase being observed in the wheat samples. These observations are in accordance with a previous study, wherein rye fermented with *L. rhamnosus*, showed a 1.4 times increase in EPC compared to non-fermented rye, whereas the increase in EPC in fermented buckwheat and barley was 1.2 times their non-fermented sample [184]. Also, Shumoy et al. [68], observed a 92-150% increase in soluble EPC in fermented teff injera (pancake) samples with the highest increase being observed at 72 h [68].

The increase in phenol content of fermented samples when baked into pancakes compared to the corresponding dough samples could be partly due to Maillard reaction products that could contribute to overall EPC [68] (as there is an increase in free sugars and proteins upon fermentation due to the action of amylases and proteases) [177].

Table 18: Extractable phenol content of different flour, dough and pancake samples at 0h, 48h and 96h of fermentation. *

	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
	Average (mg GAE/g sample, db)						
White teff	2.06 ± 0.06 c	1.94 ± 0.02 c	1.91 ± 0.09 c	2.96 ± 0.13 b	3.13 ± 0.24 b	3.76 ± 0.09 a	3.64 ± 0.05 a
Brown teff	2.60 ± 0.03 b	2.57 ± 0.04 b	2.25 ± 0.04 b	3.59 ± 0.34 a	3.64 ± 0.35 a	3.84 ± 0.23 a	3.91 ± 0.13 a
White wheat	1.06 ± 0.06 e	1.17 ± 0.13 e	1.14 ± 0.09 e	1.79 ± 0.07 d	3.09 ± 0.24 b	2.46 ± 0.05 c	3.43 ± 0.05 a
Red wheat	1.24 ± 0.12 d	1.25 ± 0.04 d	1.28 ± 0.15 d	2.17 ± 0.12 c	2.76 ± 0.13 b	2.52 ± 0.05 b	3.07 ± 0.31 a
White sorghum	0.83 ± 0.09 d	0.87 ± 0.06 d	0.81 ± 0.14 d	1.07 ± 0.06 c	1.88 ± 0.15 a	1.27 ± 0.06 b	1.86 ± 0.06 a
Lemon yellow sorghum	1.81 ± 0.18 c	1.61 ± 0.10 d	1.54 ± 0.06 d	1.92 ± 0.16 c	2.32 ± 0.08 b	2.32 ± 0.03 b	2.98 ± 0.04 a

*Data are expressed as mean ± standard deviation (n=3). Different letters in the same row indicate significant differences among the samples based on Tukey's HSD ($p<0.05$).

6.3.4.2. Effect of fermentation and baking on antioxidant potential of wheat, sorghum and teff dough and pancakes based on ABTS and ORAC assays

Different antioxidant assays are based on different reaction mechanisms. ABTS assay is based on an electron transfer mechanism, which involves a non-competitive redox reaction between the antioxidant and the oxidant. On the other hand, ORAC assay is based on a hydrogen atom transfer mechanism which involves competition between antioxidant and substrate to scavenge thermally generated peroxyl radicals from azo based compounds [172, 185]. Thus, utilization of both assays gives a more reliable determination of the antioxidant potential of the fermented samples. The antioxidant potential of the samples based on ORAC are presented in Table 19 and based on ABTS are presented in Table 20.

6.3.4.2.1. Effect of fermentation on antioxidant potential of wheat, sorghum and teff dough and pancakes based on ABTS and ORAC assays

Overall, the fermented (48 h and 96 h) dough samples showed higher antioxidant potential compared to non-fermented dough (0 h), with a gradual increase in antioxidant capacity with fermentation time (48 h to 96 h). The highest increase in ORAC was observed in fermented white teff dough samples compared to their corresponding non-fermented samples (approx. 3 times increase in 96 h fermented dough compared to non-fermented dough (0 h)) whereas the ORAC antioxidant capacity increased between 1.7 – 3 times in the 96 h dough for wheat and sorghum samples compared to 0 h dough. The highest increase in ABTS value was observed in fermented white teff dough samples compared to their corresponding non-fermented counterpart (approx. 3.4 times increase in 96 h fermented dough compared to 0 h dough) whereas the ABTS antioxidant capacity increased between

1.9 – 3 times in the 96 h fermented dough for wheat and sorghum samples compared to 0 h dough.

6.3.4.2.2. Effect of baking on antioxidant potential of wheat, sorghum and teff dough and pancakes based on ABTS and ORAC assays

Overall, fermented and baked pancake samples (48 h and 96 h) had higher antioxidant potential compared to non-fermented pancake (0 h). White/red wheat fermented pancakes showed highest ORAC value compared to their non-fermented counterparts (approx. 4.2-5.4 times increase in ORAC value in 96 h fermented pancake compared to 0 h pancake) whereas the teff and sorghum 96 h fermented pancakes showed a 2.3 – 3.5 times increase in ORAC value compared to 0 h pancakes (Table 19). White/red wheat and white teff fermented pancake samples showed highest ABTS value compared to non-fermented counterparts (approx. 3.7-3.9 times increase in 96 h pancake compared to 0 h pancake) whereas the sorghum 96 h pancake samples showed a 2.2 – 2.5 times increase in antioxidant capacity compared to 0 h pancake (Table 20). Further, the fermented pancake samples (48 h and 96 h) had higher antioxidant capacity (ORAC and ABTS) compared to corresponding dough. however, this effect was not observed in the non-fermented pancake samples (wherein the antioxidant capacity of the dough and pancakes were similar).

Thus, overall, the antioxidant potential of the fermented products increased with fermentation time, the changes in antioxidant potential varied among the different grains and baking increased the antioxidant activity of the fermented pancakes compared to corresponding fermented dough. These observations were in accordance with the increase in EPC (section 6.3.4.1.) and thus, all inferences with respect to the increase in bound polyphenols, reduced matrix effect in the fermented samples as well as formation of Maillard

reaction products could also be potential reasons for the observed increase in antioxidant potential. Furthermore, differences in the increase in antioxidant potential in pancake samples could be due to different Maillard reaction products being formed which are dependent on the total protein, the profile and content of amino acids in the different grain varieties [68, 186]. A major reason contributing to the observed changes in antioxidant capacity could also be attributed to the structural changes in polyphenols upon fermentation observed across the different grains and is discussed in section 6.3.4.3.

The observed results were in accordance with previous studies wherein there was increase in antioxidant potential in fermented buckwheat, barley, wheat and rye samples measured by FRAP assay as well as TBARS inhibition which correlated to their EPC observations with different grains showing variable increases in the antioxidant capacities [184]. Similarly, ABTS values of fermented teff injera's was higher than the non-fermented sample correlating with their TPC observations [68].

6.3.4.3. Association of structural changes observed upon fermentation in polyphenols with their antioxidant activity in wheat, teff and sorghum samples

A large portion of the variation in antioxidant potential in the grain samples and their fermented counterparts, could be attributed to the different relative concentration of diverse structural flavonoids and phenolic acids present in the grains studied (Tables A-1 - A-6). Further, in the process of fermentation these polyphenolic compounds are undergoing structural transformation that can affect the antioxidant potential (Tables A-1 - A-6).

In the teff fermented samples, there was a decrease in the C-glycoside-O-glycoside-flavonoids, O-glycoside-flavonoids and acyl derivatives of flavonoids, with a corresponding increase in mono-C-glucoside forms and the aglycone form of the corresponding flavonoids

(Table 13 & 16 and A-1, A-2). Similarly, in lemon yellow sorghum fermented samples, there was significant decrease of the O-glucoside flavanones, with a corresponding increase in the aglycone flavanone forms (Table 13, Table A-6). A study by Materska et al., isolating C-glucosides from pepper fruit found that aglycone form of luteolin and luteolin-C-glucoside showed similar and higher antioxidant potential than luteolin-6,8-di-C-glucoside whereas apigenin-6-C-glucoside-8-C-arabinoside showed lower antioxidant potential than aglycone apigenin (by both enzymatic and non-enzymatic – DPPH assays) [187]. Also, in general O-glycoside flavonoids were found to have lower TEAC values compared to their corresponding aglycone forms [188]. This could thus be a reason for the increase in antioxidant potential in teff and lemon-yellow sorghum samples upon fermentation.

In the white sorghum fermented samples, there was a significant decrease in the phenolic acid glycerol derivatives, with a corresponding increase in corresponding metabolites (dihydroferulic acid and vinylcatechol) as well as increase in bound ferulic acid. The aglycone form of apigenin and luteolin did not change in content upon fermentation (Table 14, table 17, Table A-5). Most of the phenolic acids in white sorghum were glycerol esters substituted with two phenolic acids. A study saw that DPPH scavenging capacity was in the order of feruloyl-glycerol>ferulic acid>di-feruloyl glycerol [189]. In another study, the ABTS and/or ORAC values of caffeic acid < dihydroferulic acid < ferulic acid [190], and another study saw that the antioxidant capacity of vinylcatechol < caffeic acid, thus differentially contributing to antioxidant activity [191]. Thus, though the di-substituted phenolic acid glycerols reduced upon fermentation, the increase in ferulic acid and phenolic acid metabolites could be compensating and contributing to increase in antioxidant potential in white sorghum fermented samples.

Lastly, the methyl gallate ester derivatives were increasing in all fermented grain samples, with the highest increase observed in wheat grains (Table 17, Table A-3, A-4). Thus, the methyl gallate ester derivatives could have contributed to the increase in antioxidant potential in all grain fermented samples and especially in wheat samples as no other changes in polyphenol profile was observed (Table A-3, A-4). Rice-Evans et al. observed that gallic acid methyl ester had higher TEAC value compared to o-coumaric, caffeic and ferulic acid [188].

Thus, several factors including polyphenol structural changes, pH decrease, matrix changes, release of bound polyphenols, production of polyphenol metabolites, and Maillard reaction products, could have contributed to the observed increase in extractable polyphenol content and antioxidant activity in the fermented grain samples.

Table 19: Antioxidant activity (measured by ORAC) of different flour, dough and pancake samples at 0h, 48h and 96h of fermentation. *

	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
	Average (µmol TE/g sample, db)						
White teff	43.67 ± 4.84 c	38.45 ± 4.50 c,d	32.65 ± 3.28 d	79.34 ± 6.99 b	72.72 ± 5.83 b	115.24 ± 8.44 a	112.69 ± 6.83 a
Brown teff	56.59 ± 4.99 d	54.58 ± 5.61 d,e	45.03 ± 4.73 e	93.97 ± 9.69 c	102.35 ± 11.51 b,c	113.80 ± 14.37 a	108.31 ± 4.22 a,b
White wheat	36.81 ± 5.19 d	39.41 ± 4.45 d	26.64 ± 2.86 e	61.21 ± 4.73 c	105.20 ± 9.97 b	95.74 ± 10.09 b	145.91 ± 8.89 a
Red wheat	32.36 ± 4.97 d	38.05 ± 1.92 d	24.78 ± 2.55 e	69.91± 5.11 c	86.36 ± 2.51 b	82.51 ± 3.22 b	101.85 ± 11.03 a
White sorghum	23.78 ± 3.63 c	22.21 ± 1.74 c	26.06 ± 5.39 c	39.36 ± 4.08 b	41.16 ± 6.63 b	37.21 ± 7.28 b	60.37 ± 5.84 a
Lemon yellow sorghum	40.11 ± 2.3 d,e	34.59 ± 5.15 e	34.60 ± 5.13 e	44.69 ± 2.87 c,d	53.73 ± 10.57 c	69.65 ± 6.89 b	95.02 ± 6.88 a

*Data are expressed as mean ± standard deviation (n=3). Different letters in the same row indicate significant differences among the samples based on Tukey's HSD ($p<0.05$). TE; trolox equivalents

Table 20: Antioxidant activities (measured by TEAC assay) of different flour, dough and pancake samples at 0h, 48h and 96h of fermentation. *

	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
	Average (µmol TE/g sample, db)						
White teff	26.86 ± 2.16 d	22.93 ± 1.11 e	19.70 ± 1.36 e	54.54 ± 1.65 c	54.82 ± 3.43 c	79.16 ± 1.84 a	74.29 ± 2.42 b
Brown teff	34.85 ± 0.48 c	31.15 ± 2.11 c	29.03 ± 0.65 c	66.37 ± 6.63 b	68.83 ± 4.18 b	77.55 ± 5.66 a	76.50 ± 3.34 a
White wheat	16.35 ± 0.85 d	17.32 ± 0.14 d	19.05 ± 1.00 d	39.31 ± 3.05 c	59.53 ± 7.58 b	52.68 ± 2.18 b	71.19 ± 5.36 a
Red wheat	20.58 ± 1.37 d	24.02 ± 2.96 d	20.38 ± 1.13 d	51.08 ± 3.40 c	59.16 ± 0.93 b	71.99 ± 4.99 a	76.42 ± 3.26 a
White sorghum	14.81 ± 1.02 c	14.78 ± 0.46 c	16.19 ± 0.48 c	26.10 ± 1.04 b	36.01 ± 2.56 a	28.23 ± 1.86 b	36.61 ± 0.06 a
Lemon yellow sorghum	22.68 ± 2.08 d	20.34 ± 1.91 d	19.87 ± 1.66 d	29.12 ± 1.22 c	36.54 ± 2.28 b	39.06 ± 1.71 b	49.89 ± 1.20 a

*Data are expressed as mean ± standard deviation (n=3). Different letters in the same row indicate significant differences among the samples based on Tukey's HSD ($p<0.05$). TE; trolox equivalents

6.4. Conclusion

This study for the first time demonstrates the effect of both fermentation and baking on flavonoids in whole grain wheat, teff and sorghum grains. Overall outcomes on different flavonoids forms identified in the grains showed that di-C-linked and mono-C-linked glycoside flavonoids were stable to both fermentation and baking. On the other hand, phenolic acids were metabolized to corresponding small compound metabolites and O-linked-glycoside-flavonoids as well as acyl derivatives of flavone-C-glycosides were metabolized to corresponding aglycone forms. Fermentation also led to the release of bound phenolic acids, ferulic acid and methyl gallate derivatives. In general, all the flavonoids were stable when baked, and irrespective of grain type, the effect of fermentation on flavonoid/phenolic acid structure was similar. All fermented dough and pancake samples had higher antioxidant potential compared to non-fermented counterparts. Also, the fermented pancake samples showed higher antioxidant potential compared to the corresponding dough. Thus, fermentation of whole grains altered the structure and content of different polyphenols in ways that could improve their bioavailability and bioactivity from respective products. Further studies with specific *lactobacilli* strains and strains capable of cleaving flavonoid-C-linkages are necessary to confirm the effects observed in this study.

7. CONCLUSIONS

7.1. Summary

Using Caco-2 model, we demonstrated that combination of cereal flavones and pulse flavonols increases their absorption. Reduced phase 2 metabolism and synergistic inhibition of ABC membrane transporter activity contributed to this effect. The structure of flavonoids plays a vital role in their enhanced bioavailability in combination, as combination of apigenin or quercetin with the flavanone - naringenin (flavonoid lacking C2=C3 double bond conjugation), did not affect absorption of the compounds. Thus, cereals and pulses, if strategically combined can be used to produce novel functional food formulations providing enhanced health benefits.

Profiling different whole grains revealed cereals to be a major source of the relatively rare flavone class of compounds varying greatly in their conjugation type to sugar residues. Majority of the compounds were identified for the first time in the different cereal grains studied, further emphasizing the need to thoroughly profile flavonoids in cereal grains based on robust methodologies. Different types of whole grain sorghum (white, lemon yellow) were mainly a source of flavone aglycones, O-linked glycoside flavonoids (flavanones) and phenolic acid derivatives. On the other hand, wheat was mainly a source of di-C-linked flavone glycosides, and teff and rye contained a mixture of mono-C-linked, di-C-linked, O-linked and O-glycoside-C-glycoside flavone and their acyl derivatives. This variation in structural profile of flavones among the cereal grains, especially the nature of glycosylation (O-linkage vs C-linkage) could translate to differences in their bioavailability, bioactivity, as well as interaction with pulse flavonoids.

Further, processing of the cereal grains could have differential effects on the different flavonoid structures. Red wheat and brown teff showed the presence of procyanidins in an unextractable form, which is a likely contributor to the color of these grains. It is important to establish the structure and profile of the procyanidins as present in these grains, and their nutritional impact, especially with respect to colon health.

The process of fermentation led to metabolism of flavonoid-O-glycosides to their aglycone forms and did not metabolize aglycone flavonoid forms. This could have a significant impact on bioavailability of flavonoids from grains containing O-glycosyl-flavonoids/aglycones such as lemon yellow and white sorghum, white teff, brown teff and rye grains (as aglycone forms show higher absorption potential in the intestine compared to corresponding glycosyl forms).

However, the process of fermentation did not metabolize C-linked glycosyl flavonoids (both mono-C and di-C-linkages). In general, the C-linked flavonoids show much reduced absorption in the small intestine compared to aglycone and O-linked glycosides. This could significantly impact the bioavailability of flavonoids especially from wheat grains (which exclusively contained di-C-linked flavones) as well as rye and teff grains. Thus, there is a need to explore strategies to enhance the bioavailability of C-linked flavones. A potential strategy could be to ferment using bacteria capable of cleaving the C-linked glycosyl linkage (e.g. *Eubacterium cellulosolvens*), thereby making available the aglycone form of the flavones. The use of this bacteria could further lead to breakdown of cell wall matrix in whole grains due to their cellulase activity. This could aid in

increased release of bound polyphenols increasing their bioaccessibility. Also, it could improve texture of whole grain products by enhancing water binding properties.

Lastly, fermentation metabolized the phenolic acids glycerol esters to mainly dihydroferulic acid and vinyl catechol. The process also led to the release of bound polyphenols in the fermented products. Thus, fermentation could help improve the bioaccessibility and bioavailability of phenolic acids from grains. Baking the fermented dough of different grains into pancakes, did not change the content of the different polyphenol forms studied indicating their stability to heat in the system studied. Fermentation increased the antioxidant potential of the grain products in comparison to the non-fermented products.

Thus, cereals and pulses present complementary structures of flavonoids that could enhance their bioavailability and hence potential bioactive properties. Further, fermentation could be used as a strategic processing aid in enhancing the bioavailability and bioactive properties of flavonoids from whole grain products.

Limitations of research

Utilization of the Caco-2 cell monolayer model, helps define the mechanisms of bioavailability of cereal-pulse flavonoid combinations in a model mimicking the properties of the small intestine. However, it is further important to understand complex interactions as could be present in an *in vivo* system on overall bioavailability (absorption and metabolism) of the flavonoid combinations (e.g. potential cross talk of liver and colon metabolites on absorption properties of compounds in the small intestine). Further, it is

important to understand the eventual bioactivity of the flavonoid combinations and their metabolites in *in vivo* animal models and human clinical trials.

Our study only utilized pure compounds to model mechanisms of bioavailability of cereal-pulse flavonoid combinations. However, the effects of consumption of cereals and pulses as whole food on flavonoid bioavailability and bioactivity needs to be investigated. This is because the bioavailability and bioactive properties could be different due to potential interactions from other compounds present in the whole food, food matrix-polyphenol interactions, differences on compound bioavailability based on processing methods used, etc.

The process of fermentation followed in this study was based on natural fermentation. However, development of standardized microbial mixtures under controlled conditions of use that can not only efficiently reproduce the observed effects on the different polyphenol structures as observed in our study, but also improve the bioavailability of C-linked flavonoids need to be explored. This could aid in development of novel functional foods offering enhanced health benefits. The subsequent effects on food product quality in terms of aroma, texture and taste will need to be evaluated. Further, it is important to determine the thermal stability of the flavonoids (especially the C-linked forms, which are less studied) to harsher processing techniques (e.g. prolonged baking conditions in the process of bread making, extrusion, frying). Subsequent impact of these processing conditions on flavonoid bioavailability and bioactivity needs to be determined.

7.2. Recommendations for future work

1. Investigate absorption, metabolism and bioactivity of cereal-pulse flavonoid combinations in *in vivo* animal models and human clinical trials.
2. Determine effect of cereal-pulse flavonoid combinations on colon microbiota and colon health.
3. Explore fermentation strategies (use of specific microbial populations) to enhance the bioavailability and bioactivity of flavonoids from whole grain products.
4. Determine effects on bioavailability and thermal stability of different flavonoid structures (especially C-linked forms) to different processing conditions (e.g. baking into breads, extrusion, frying).

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APPENDIX A

EFFECT OF FERMENTATION AND BAKING ON CONCENTRATION OF POLYPHENOLS IN WHEAT, SORGHUM AND TEFF GRAIN DOUGH AND PANCAKE SAMPLES

Table A-1: Concentration (µg/g, dry weight basis) of polyphenols in white teff non-fermented (0 h) and fermented (48 h and 96 h) dough and pancake samples[a.]

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
		Average µg/g of sample, db						
White Teff – Mixture of di-C-glycosides, mono-C-glycosides, O-glycosides and acyl-C-glycoside flavones (mainly apigenin)								
1	Apigenin-6,8-C-diglucoside[b]	69.7 ± 3.0	56.7 ± 1.1	37.2 ± 1.1	59.0 ± 3.6	51.8 ± 3.4	27.8 ± 6.4	28.8 ± 1.8
2	Apigenin-8-C-glucosyl-7-O-glucoside[b]	263.5 ± 8.4	215.1 ± 2.2	171.5 ± 2.4	189.7 ± 5.7	160.0 ± 4.3	148.7 ± 5.1	129.8 ± 2.6
3	Apigenin-6-C-glucosyl-2”-O-glucoside[b]	207.1 ± 9.6	166.3 ± 2.2	129.3 ± 2.1	148.6 ± 7.0	129.9 ± 4.6	99.9 ± 2.3	91.5 ± 1.1
4	Apigenin-6-C-galactosyl-8-C-arabinoside[b]	30.1 ± 0.8	36.2 ± 1.1	31.3 ± 0.9	47.1 ± 3.1	44.2 ± 2.9	53.6 ± 2.3	51.0 ± 1.4
5	Apigenin-6-C-glucosyl-8-C-arabinoside[b]	44.3 ± 1.6	39.7 ± 0.1	31.0 ± 1.1	42.1 ± 3.3	35.1 ± 1.6	44.5 ± 2.5	39.5 ± 1.6
6	Apigenin-8-C-glucoside[b]	181.4 ± 11.6	141.3 ± 3.9	133.7 ± 4.0	231.5 ± 10.1	227.3 ± 9.4	254.9 ± 8.0	253.9 ± 4.7
7	Apigenin-6-C-glucoside[b]	19.3 ± 0.7	14.7 ± 0.4	13.1 ± 1.1	111.3 ± 4.7	107.5 ± 4.3	144.8 ± 3.6	137.1 ± 1.7
8	Apigenin-7-O-Me-6/8-C-glucoside[b]	24.0 ± 1.2	23.3 ± 0.2	17.4 ± 1.2	29.1 ± 2.0	26.8 ± 1.2	28.2 ± 1.9	26.1 ± 0.7
9	Apigenin-7-O-neohesperidoside[b]	105.8 ± 4.3	78.8 ± 2.0	79.4 ± 5.2	11.7 ± 1.7	17.1 ± 1.5	2.5 ± 0.2	8.0 ± 1.6
10	Apigenin-7-O-(6”-syringyl)glucosyl-6-C-glucoside[b]	81.6 ± 3.6	66.8 ± 1.7	57.9 ± 3.3	52.9 ± 4.9	36.6 ± 2.0	28.9 ± 1.4	25.9 ± 2.8

Table A-1 continued

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
		Average µg/g of sample, db						
11	Apigenin-7-O-(6"-syringyl)arabinosyl-8-C-glucoside[b]	183.3 ± 8.5	128.0 ± 2.6	118.0 ± 3.7	6.4 ± 0.3	4.8 ± 0.4	4.3 ± 0.3	4.3 ± 0.3
12	Apigenin-8-C-(6"-acetyl) glucoside[b]	60.9 ± 2.7	26.4 ± 0.9	42.5 ± 1.7	1.8 ± 0.1	4.7 ± 0.6	ND	2.6 ± 0.1
13	7-O-methyl apigenin-8-C-(2"-succinyl) arabinoside[b]	71.8 ± 3.7	49.7 ± 0.9	47.8 ± 2.1	0.5 ± 0.6	0.4 ± 0.1	ND	ND
29	Apigenin[d]	2.6 ± 0.8	6.4 ± 1.8	4.0 ± 0.8	16.1 ± 3.6	16.9 ± 3.6	21.5 ± 4.5	22.0 ± 4.6
	Total	**1345.4 ± 59.5 A**	**1049.2 ± 20.2 B**	**914.2 ± 27.8 C,D**	**947.8 ± 45.6 C**	**863.0 ± 37.3 D,E**	**860.8 ± 34.5 D,E**	**820.5 ± 23.3 E**
Phenolic acids								
27	Dicoumaroylglycerol[j]	13.8 ± 2.5	8.9 ± 0.6	8.5 ± 0.6	ND	ND	ND	ND
28	p-coumaroyl-feruloyl-glcerol[j]	15.0 ± 2.2	8.7 ± 0.7	8.5 ± 0.4	ND	ND	ND	ND
60	Methyl gallate derivative [k]	ND	ND	ND	32.4 ± 0.8	26.8 ± 0.6	37.3 ± 0.4	31.1 ± 1.0
61	Methyl gallate derivative [k]	ND	ND	ND	33.7 ± 1.2	25.8 ± 1.2	102.5 ± 1.4	58.4 ± 1.9
	Total	**28.8 ± 4.7 E**	**17.6 ± 1.3 F**	**16.9 ± 1.1 F**	**66.1 ± 1.9 C**	**52.6 ± 1.8 D**	**139.7 ± 1.8 A**	**89.5 ± 2.9 B**

[a]Data are based on UPLC quantification of identified peaks and are expressed as mean ± standard deviation (n=3). [b]As apigenin-7-O-glucoside equivalents. [d] As apigenin equivalents..[j] as coumaric acid equivalents. [k] as gallic acid equivalents. ND: not detected. Peaks that were not structurally identified are not included. Different capital letters indicate significant difference among all the samples in total content ($p<0.05$).

Table A-2: Concentration (µg/g, dry weight basis) of polyphenols in brown teff non-fermented (0 h) and fermented (48 h and 96 h) dough and pancake samples[a.]

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
		Average µg/g of sample, db						
Brown Teff – Mixture of di-C-glycosides, mono-C-glycosides, O-glycosides and acyl-C-glycoside flavones (mainly luteolin)								
14	Luteolin-6,8-di-C-glucoside[c]	23.1 ± 2.2	24.2 ± 2.0	18.2 ± 1.2	15.2 ± 2.64	35.4 ± 6.1	0.5 ± 0.8	25.2 ± 21.8
15	Luteolin -8-C-glucosyl-7-O-glucoside isomer[c]	356.0 ± 45.1	343.7 ± 12.5	257.4 ±4.4	224.9 ± 21.4	194.1 ± 13.8	172.4 ± 12.3	178.4 ± 12.4
16	Luteolin -6-C-glucosyl-7-O-glucoside isomer[c]	342.7 ± 5.1	329.9 ± 11.1	234.9 ± 1.3	232.3 ± 20.2	207.9 ± 5.8	148.0 ± 6.6	153.8 ± 5.1
17	Luteolin-8-C-glucosyl-7-O-neohesperidoside[c]	56.4 ± 6.4	112.8 ± 7.1	44.2 ± 2.7	64.0 ± 17.2	75.1 ± 4.8	122.3 ± 7.7	104.5 ± 6.4
18	Apigenin-6-C-glucosyl-2”-O-glucoside[b]	32.4 ± 1.8	32.1 ± 1.4	23.1 ± 0.7	13.9 ± 10.3	26.3 ± 1.4	6.4 ± 2.1	24.2 ± 1.6
19	Luteolin-8-C-glucoside[c]	299.8 ± 13.3	274.2 ± 9.4	233.8 ± 3.6	516.5 ± 26.9	504.2 ± 26.4	512.4 ± 35.8	539.5 ± 16.7
20	Chrysoeriol -8-C-(2”-O-glucosyl)glucoside[c]	80.5 ± 1.7	79.3 ± 4.6	38.8 ± 16.5	27.5 ± 3.4	26.4 ± 1.0	22.0 ± 1.0	24.1 ± 0.6
21	Apigenin-8-C-glucoside[b]	14.3 ± 0.9	19.6 ± 2.0	10.0 ± 0.6	53.1 ± 4.1	56.3 ± 3.0	53.6 ± 3.7	57.9 ± 1.8
22	Luteolin-7-O-glucoside[c]	78.2 ± 35.8	56.5 ± 3.9	50.8 ± 3.1				
23	Luteolin-7-O-neohesperidoside[c]	108.8 ± 21.0	112.4 ± 5.9	97.6 ± 3.7	21.8 ± 1.6	22.9 ± 1.1	21.5± 1.4	24.9 ± 1.0
24	Luteolin-7-O-(6”-syringly)glucosyl-6-C-glucoside[c]	152.5 ± 115.7	88.5 ± 9.9	60.8 ± 6.6	49.0 ± 4.2	45.8 ± 2.9	44.9 ± 2.6	43.9 ± 1.5
25	Luteolin-7-O-(2”-syringyl)arabinosyl-6-C-glucoside[c]	210.4 ± 136.7	226.2 ± 9.1	192.2 ± 3.3	11.7 ± 2.5	14.3 ± 1.3	7.5 ± 0.3	7.0 ± 0.3

Table A-2 continued

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
					Average µg/g of sample, db			
26	Luteolin-8-C-(6”-diacetyl)glucoside[c]	54.2 ± 3.5	45.7 ± 2.8	ND	ND	ND	ND	ND
30	Luteolin[e]	ND	ND	ND	6.6 ± 2.8	8.6 ± 2.2	7.4 ± 1.5	9.2 ± 1.9
	Total	**1809.5 ± 73.4 A**	**1745.0 ± 79.6 A**	**1303.2 ± 8.2 B**	**1236.5 ± 75.2 B**	**1217.4 ± 55.8 B,C**	**1118.7 ± 74.2 C**	**1192.5 ± 44.0 B,C**
Phenolic acids								
27	Dicoumaroylglycerol[j]	7.6 ± 0.5	6.2 ± 1.1	6.5 ± 0.4	ND	ND	ND	ND
28	p-coumaroyl-feruloyl-glcerol[j]	10.9 ± 0.6	7.1 ± 0.8	7.1 ± 0.4	ND	ND	ND	ND
60	Methyl gallate derivative [k]	ND	ND	ND	135.2 ± 8.0	106.0 ± 4.6	137.3 ± 7.4	118.7 ± 5.5
61	Methyl gallate derivative [k]	ND	ND	ND	42.4 ± 0.7	31.7 ± 1.7	82.7 ± 4.8	57.6 ± 2.2
	Total	**18.5 ± 1.1 D**	**13.3 ± 1.9 D**	**13.6 ± 0.7 D**	**177.6 ± 8.7 B**	**137.7 ± 6.3 C**	**220.0 ± 12.2 A**	**176.3 ± 7.7 B**

[a]Data are based on UPLC quantification of identified peaks and are expressed as mean ± standard deviation (n=3). [b]As apigenin-7-O-glucoside equivalents. [c]As luteolin-7-O-glucoside equivalents. [e] As luteolin equivalents. .[j] as coumaric acid equivalents. [k] as gallic acid equivalents. ND: not detected. Peaks that were not structurally identified are not included. Different capital letters indicate significant difference among all the samples in total content ($p<0.05$).

Table A-3: Concentration (μg/g, dry weight basis) of polyphenols in white wheat non-fermented (0 h) and fermented (48 h and 96 h) dough and pancake samples[a.]

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
					Average μg/g of sample, db			
White wheat								
31	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	29.8 ± 1.1	24.1 ± 1.7	20.4 ± 1.2	22.8 ± 0.3	25.6 ± 4.9	23.8 ± 3.7	26.4 ± 0.6
32	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	52.2 ± 2.8	46.7 ± 2.4	35.5 ± 1.2	40.7 ± 0.4	35.8 ± 2.0	41.4 ± 3.7	45.3 ± 2.2
33	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	3.6 ± 0.3	2.3 ± 0.9	3.0 ± 1.3	3.1 ± 0.1	3.7 ± 0.2	6.7 ± 2.6	10.4 ± 0.4
34	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	3.6 ± 0.2	2.7 ± 1.1	2.6 ± 1.5	1.8 ± 0.1	5.7 ± 0.3	4.2 ± 2.4	13.9 ± 1.4
35	Apigenin-6-C-sinapoylpentoside-8-C-hexoside[b]	18.5 ± 0.4	10.1 ± 3.2	8.2 ± 2.8	5.5 ± 0.1	5.6 ± 0.5	14.5 ± 4.1	15.3 ± 0.2
36	Apigenin-8-C-sinapoylpentoside-8-C-hexoside[b]	33.1 ± 4.0	21.6 ± 4.3	19.5 ± 2.7	16.3 ± 0.4	12.8 ± 0.7	18.7 ± 2.4	24.2 ± 0.0
	Total	**140.8 ± 8.4 A**	**107.5 ± 13.3 B,C**	**89.3 ± 10.7 C**	**90.2 ± 0.8 B,C**	**89.2 ± 8.6 C**	**109.3 ± 18.8 B**	**135.5 ± 1.0 A**
Phenolic acids								
60	Methyl gallate derivative [k]	ND	ND	ND	154.4 ± 2.6	146.3 ± 7.9	90.0 ± 6.3	122.3 ± 0.7
61	Methyl gallate derivative [k]	ND	ND	ND	17.3 ± 1.7	20.6 ± 3.6	40.5 ± 2.4	31.3 ± 0.2
	Total				**171.6 ± 4.3 A**	**166.9 ± 4.3 A**	**130.5 ± 6.5 C**	**153.6 ± 0.6 B**

[a]Data are based on UPLC quantification of identified peaks and are expressed as mean ± standard deviation (n=3). [b]As apigenin-7-O-glucoside equivalents. [k] as gallic acid equivalents. ND: not detected. Peaks that were not structurally identified are not included. Different capital letters indicate significant difference among all the samples in total content ($p<0.05$).

Table A-4: Concentration (µg/g, dry weight basis) of polyphenols in red wheat non-fermented (0 h) and fermented (48 h and 96 h) dough and pancake samples[a.]

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
		Average µg/g of sample, db						
Red wheat								
31	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	44.0 ± 2.4	36.2 ± 0.5	35.7 ± 0.5	32.8 ± 3.2	31.1 ± 0.5	33.4 ± 1.1	33.0 ± 2.1
32	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	78.0 ± 4.0	74.0 ± 0.6	60.6 ± 0.9	65.2 ± 3.1	56.0 ± 2.5	70.3 ± 3.1	70.3 ± 2.3
33	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	1.1 ± 0.2	0.6 ± 0.1	0.8 ± 0.2	4.1 ± 0.5	3.9 ± 0.2	5.9 ± 0.2	6.5 ± 0.3
34	Apigenin-6-C-pentoside-8-C-hexoside isomers[b]	2.2 ± 0.5	1.9 ± 0.0	2.4 ± 0.4	2.0 ± 0.1	3.1 ± 0.3	2.0 ± 0.3	2.8 ± 0.3
35	Apigenin-6-C-sinapoylpentoside-8-C-hexoside[b]	14.4 ± 1.7	11.1 ± 0.9	11.4 ± 0.7	8.2 ± 1.1	8.0 ± 1.3	7.4 ± 0.6	8.6 ± 0.4
36	Apigenin-8-C-sinapoylpentoside-8-C-hexoside[b]	37.9 ± 3.5	29.7 ± 0.5	32.1 ± 1.9	26.3 ± 0.8	23.9 ± 3.4	22.2 ± 2.0	26.1 ± 0.6
	Total	**177.59 ± 11.5 A**	**153.5 ± 0.9 B**	**143.0 ± 4.2 B,C**	**138.6 ± 6.7 C**	**125.9 ± 1.8 D**	**141.2 ± 6.2 C**	**147.3 ± 4.6 B,C**
Phenolic acids								
60	Methyl gallate derivative [k]	ND	ND	ND	194.1 ± 6.1	202.8 ± 9.9	231.7 ± 8.7	233.0 ± 5.9
61	Methyl gallate derivative [k]	ND	ND	ND	12.5 ± 0.5		29.9 ± 1.5	21.5 ± 0.9
	Total				**206.6 ± 5.6 B**	**202.8 ± 9.9 B**	**261.6 ± 10.2 A**	**254.6 ± 6.8 A**

[a]Data are based on UPLC quantification of identified peaks and are expressed as mean ± standard deviation (n=3). [b]As apigenin-7-O-glucoside equivalents. [k] as gallic acid equivalents. ND: not detected. Peaks that were not structurally identified are not included. Different capital letters indicate significant difference among all the samples in total content ($p<0.05$).

Table A-5: Concentration (µg/g, dry weight basis) of polyphenols in white sorghum non-fermented (0 h) and fermented (48 h and 96 h) dough and pancake samples[a].

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
		Average µg/g of sample, db						
White sorghum – Flavone aglycones								
30	Luteolin[e]	28.0 ± 6.0	25.6 ± 5.4	30.9 ± 5.9	27.3 ± 7.2	27.2 ± 7.3	25.3 ± 4.6	25.8 ± 5.2
29	Apigenin[d]	60.3 ± 10.2	62.5 ± 12.7	73.0 ± 12.7	62.1 ± 12.8	65.1 ± 16.4	54.9 ± 8.1	56.2 ± 8.8
	Total	**88.3 ± 16.2**	**88.1 ± 18.1**	**103.9 ± 18.6**	**89.4 ± 20.1**	**92.3 ± 23.7**	**80.2 ± 12.7**	**82.0 ± 14.0**
Phenolic acids								
37	2-O-caffeoylglycerol-O-glucoside [f]	32.3 ± 1.0	38.9 ± 1.0	40.9 ± 0.0	8.9 ± 2.4	14.0 ± 0.2	ND	ND
38	1-O-caffeoylglycerol-O-glucoside [f]	38.2 ± 0.1	48.3 ± 0.4	52.4 ± 1.1	10.8 ± 1.1	9.4 ± 0.0	11.3 ± 0.2	14.3 ± 0.3
39	2-O-caffeoylglycerol [f]	25.2 ± 1.2	16.4 ± 0.4	22.6 ± 0.2	5.6 ± 1.3	7.4 ± 0.3	ND	ND
40	1-O-caffeoylglycerol [f]	31.0 ± 1.7	21.9 ± 0.7	28.9 ± 0.7	4.1 ± 1.1	2.5 ± 0.1		
41	N, N' - Dicaffeoylspermidine [f]	31.1 ± 0.8	38.5 ± 0.9	39.9 ± 1.0	13.0 ± 5.1	19.2 ± 0.7	10.0 ± 0.5	8.4 ± 1.1
42	N, N' - Dicaffeoylspermidine [f]	29.4 ± 1.9	21.6 ± 1.3	26.2 ± 3.9	ND	ND	ND	ND
43	Dihydroferulic acid[i]	ND	ND	ND	6.8 ± 0.0	9.7 ± 0.5	4.7 ± 0.1	4.8 ± 3.7
44	Ferulic acid[i]	21.2 ± 1.1	27.7 ± 0.5	28.8 ± 1.6	73.6 ± 4.4	88.8 ± 3.4	79.9 ± 3.2	72.3 ± 2.1
45	Vinyl catechol	ND	ND	ND	D	D	D	D
46	Dicaffeoylglycerol [f]	45.5 ± 4.3	40.0 ± 2.8	50.4 ± 1.1	13.4 ± 1.5	28.3 ± 2.0	ND	ND
47	Dicaffeoylglycerol [f]	137.3 ± 10.4	121.5 ± 9.3	139.0 ± 4.1	27.2 ± 3.7	17.4 ± 2.4	ND	ND
48	p-coumaroyl-caffeoyl-glycerol isomer[j]	140.6 ±20.9	133.5 ± 3.5	156.3 ± 2.5	33.3 ± 6.4	74.6 ± 5.4	ND	ND
49	Feruloyl-caffeoyl-glycerol[i]	85.3 ± 3.0	78.2 ± 11.5	83.6 ± 4.2	6.9 ± 2.3	6.3 ± 8.8	ND	ND
61	Methyl gallate derivative [k]	ND	ND	ND	157.2 ± 5.7	110.5 ± 1.6	146.0 ± 2.1	99.4 ± 2.8
	Total	**617.0 ± 38.3 B**	**586.6 ± 22.6 B**	**669.2 ± 15.4 A**	**358.6 ± 7.7 C**	**277.4 ± 4.6 C**	**251.8 ± 5.0 D**	**199.2 ± 6.4 E**

[a]Data are based on UPLC quantification of identified peaks (Table 1) and are expressed as mean ± standard deviation (n=3). [d] As apigenin equivalents. [e] As luteolin equivalents. [f] As caffeic acid equivalents. [i] As ferulic acid equivalents.[j] as coumaric acid equivalents. [k] as gallic acid equivalents. ND: not detected. D: detected but not quantified. Peaks that were not structurally identified are not included. Different capital letters indicate significant difference among all the samples in total content ($p<0.05$).

Table A-6: Concentration (μg/g, dry weight basis) of polyphenols in lemon-yellow sorghum non-fermented (0 h) and fermented (48 h and 96 h) dough and pancake samples[a.]

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
				Average μg/g of sample, db				
Lemon Yellow – Flavanone-O-glycosides								
50	Eriodictyol-7-O-galactoside[g]	68.7 ± 1.4	49.5 ± 3.4	95.2 ± 2.9	ND	ND	ND	ND
51	Eriodictyol-5-O-galactoside[g]	91.3 ± 0.9	66.9 ± 2.8	141.5 ± 2.6	ND	ND	ND	ND
52	Eriodictyol-7-O-glucoside[g]	280.9 ± 6.6	186.6 ± 16.1	177.9 ± 11.4	126.4 ± 1.2	143.2 ± 0.9	123.2 ± 4.5	199.9 ± 16.9
53	Eriodictyol-5-O-glucoside[g]	327.4 ± 13.8	250.2 ± 1.7	172.0 ± 11.6	ND	ND	ND	ND
54	Naringenin-7-O-galactoside[h]	46.6 ± 1.3	37.3 ± 2.2	56.4 ± 0.8	ND	ND	ND	ND
55	Naringenin-5-O-galactoside[h]	161.5 ± 5.1	153.9 ± 8.2	125.7 ± 9.0	115.8 ± 18.4	95.23 ± 16.80	122.61 ± 6.06	182.38 ± 5.37
56	Eriodictyol[g]	85.1 ± 12.1	93.2 ± 15.6	81.0 ± 12.5	239 ± 10	242 ± 13	252 ± 5	292 ± 16
57	Naringenin[h]	37.2 ± 4.8	36.2 ± 6.4	29.7 ± 4.5	70 ± 10	78 ± 5	73 ± 0	87 ± 6
	Total	**1098.7 ± 42.5 A**	**873.9 ± 54.5 B**	**879.3 ± 27.0 B**	**551 ± 12 D**	**558 ± 31 D**	**571 ± 5 D**	**761 ± 41 C**
Flavone-O-glycoside chalcones								
58	Eriodictyol-7-O-galactoside chalcone[g]	29.6 ± 2.5	21.3 ± 7.6	80.5 ± 14.9	4.7 ± 1.8	12.1 ± 2.1	4.3 ± 0.9	14.2 ± 6.4
59	Naringenin-7-O-galactoside chalcone[h]	28.9 ± 7.5	19.0 ± 7.8	24.4 ± 7.2	5.6 ± 2.9	12.0 ± 2.4	3.5 ± 2.1	14.3 ± 2.7
	Total	**58.5 ± 9.6 B**	**40.4 ± 15.5 B,C**	**104.9 ± 22.1 A**	**10.3 ± 4.7 D,E**	**24.0 ± 4.0 C,D,E**	**7.8 ± 2.8 E**	**28.5 ± 8.8 C,D**

Table A-6 continued

Peak no.	Proposed identification	Flour	Dough 0h	Pancake 0h	Dough 48h	Pancake 48h	Dough 96h	Pancake 96h
				Average µg/g of sample, db				
Phenolic acids								
38	1-O-caffeoylglycerol-O-glucoside [f]	17.3 ± 0.5	29.2 ± 1.8	21.6 ± 2.2	ND	ND	ND	ND
39	2-O-caffeoylglycerol [f]	10.1 ± 0.7	0.3 ± 0	10.1 ± 0.5	ND	ND	ND	ND
40	1-O-caffeoylglycerol [f]	10.8 ± 0.9	0.7 ± 0.1	9.3 ± 0.2	ND	ND	ND	ND
46	Dicaffeoylglycerol [f]	36.0 ± 7.5	40.9 ± 26.1	26.5 ± 4.0	ND	ND	ND	ND
47	Dicaffeoylglycerol [f]	56.5 ± 10.1	42.8 ± 12.1	39.9 ± 7.1	ND	ND	ND	ND
44	Ferulic acid[i]	ND	ND	ND	ND	ND	9.8 ± 1.5	17.4 ± 0.8
61	Methyl gallate derivative [k]	ND	ND	ND	47.1 ± 0.9	42.9 ± 0.2	66.4 ± 1.1	54.3 ± 0.2
	Total	**130.9 ± 18.2 A**	**113.8 ± 37.4 A**	**107.4 ± 11.5 A**	**47.1 ± 0.9 C**	**42.9 ± 0.2 C**	**76.3 ± 2.5 B**	**73.0 ± 1.1 B**

[a]Data are based on UPLC quantification of identified peaks (Table 1) and are expressed as mean ± standard deviation (n=3). [g] As eriodictyol equivalents. [h] As naringenin equivalents. [f] As caffeic acid equivalents. [i] As ferulic acid equivalents. [k] as gallic acid equivalents Peaks that were not structurally identified are not included. ND: not detected. Different capital letters indicate significant difference among all the samples in total content ($p<0.05$).