Bioaccessibility of Ferulic Acid in Barley

By

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ABSTRACT

Whole grain cereals are sources of starch, proteins, lipids, and dietary fibre, along with micronutrients and phytochemicals. The most consumed cereal grain in Canada is wheat, however, the consumption of hulless (food-grade) barley varieties is increasing. The consumption of whole grains has been associated with health benefits including preventing cardiovascular disease and cancer. Contributing to the benefits are phenolic acids which exhibit antioxidant and anti-inflammatory effects. The major phenolic acid in whole grains is ferulic acid. Ferulic acid is predominately found in bound form and is concentrated in the outer bran layers of the grain, the aleurone layer, and the germ. Bound ferulic acid is covalently bonded to cell wall carbohydrates such as arabinoxylans. The bound form cannot be absorbed in the small intestine and thus is not bioaccessible. An understanding of ferulic acid bioaccessibility helps determine its bioavailability and ultimately its potential for providing health benefits. Therefore, the objectives of this research were to 1) characterize the phenolic acid composition of raw, cooked and digested barleys, 2) determine the ferulic acid bioaccessibility and determine when the ferulic acid is primarily released, and 3) examine factors which may influence the bioaccessibility of ferulic acid including bran layer thickness, and arabinoxylan content and structural characteristics. Firstly, the phenolic acid composition of four hulless barley varieties were examined using HPLC. Boiling enhanced the extractability of bound phenolic acids and had little effect on the level of free phenolic acids. A static digestion model was used to simulate gastrointestinal digestion and determine the bioaccessibility of phenolic acids. Ferulic and *p*-coumaric acids had the highest bioaccessibilities, up to 173% and 135% respectively. Ferulic acid bioaccessibility was significantly greatest in the variety Peru-35. Two varieties, Peru-35 and Roseland were selected for the remaining experiments. The level of free and bound ferulic acid varied throughout digestion. The release of ferulic acid

was strongly influenced by digestive enzymes and minimally affected by pH. The release of ferulic acid primarily occurred during the final hour of small intestinal digestion. The variation observed between varieties suggests that the grain matrix influences the bioaccessibility of ferulic acid by determining the interactions ferulic acid has with other grain components (and thus its stability) within the digestive environment. Bran thickness may also influence ferulic acid bioaccessibility but further research is needed. The barleys were sequentially pearled, and the fractions obtained were analyzed in terms of arabinoxylan content and A/X ratio by gas chromatography, and ferulic and *p*-coumaric acid content by HPLC. The amount of ferulic and *p*-coumaric acids was greatest in the outermost fraction and progressively decreased towards the endosperm-containing fraction. Similarly, the arabinoxylan content was greatest in the outermost layer and was positively correlated with bound phenolic acid content. The A/X ratio varied among fractions and correlated with bound *p*-coumaric content. Additionally, a polyamine, *N*1, *N*8-dicaffeoyl spermidine, was identified in the free extracts which has not been reported before in whole grain barley and may provide additional anti-inflammatory and antioxidant effects. *N*1, *N*8-dicaffeoyl spermidine was more evenly distributed throughout the grain kernel than the phenolic acids. Its abundance was greatest in the aleurone-containing fraction of Peru-35, and the pericarp-containing fraction of Roseland. Since phenolic acids are known to interact with polyamines, proteins and carbohydrates, further research examining the relationship between *N*1, *N*8- dicaffeoyl spermidine, arabinoxylans and phenolic acids within the barley grain matrix is needed. This research is the first to investigate the bioaccessibility of ferulic acid in hulless barley. The research provides a foundation for additional research on determining factors which influence ferulic acid bioaccessibility in grains. Knowledge gained from this research facilitates the selection of grain fractions for functional food development.

Dedication

To my family, for their endless love and support.

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Thesis Format

This thesis is presented in the manuscript format. The referencing style used is that of the Food Chemistry journal. The thesis has six chapters of which Chapters 1 and 2 provide an introduction and literature review. There are three experimental chapters (Chapters 3, 4 and 5). Chapter 6 summarizes the research and provides an overall discussion and conclusion, as well as recommendations for future research.

Contributions of Authors

Experimental Chapters

Chapter 3: Bioaccessibility of phenolic acids in Canadian hulless barley varieties

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- My role for this research chapter included: formal analysis, data curation, investigation, methodology, and writing of the original draft.
- Dr. Franklin Apea-Bah: Methodology, writing-reviewing and editing.
- Dr. Polyanna Silveira Hornung: Methodology, writing-reviewing and editing.
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The Canadian Grain Commission, Winnipeg, conducted the pearling to obtain the pearled fractions, and the analysis of arabinoxylans using gas chromatography. I thank Dr. Marta Izydorczyk and the Grain Research Laboratory of the Canadian Grain Commission for their assistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1.

Introduction

1. Introduction

Whole grains have received much attention due to the health benefits associated with their regular consumption. Regular consumption of whole grains has been associated with blood glucose management, reduced risk of obesity and cardiovascular diseases (Cho, Qi, Fahey, & Klurfeld, 2013), and promotion of gut health (Foerster et al., 2014). Wheat is the most consumed cereal grain in Canada with many of the population's staple foods comprising of wheat products including bread, pasta and crackers. However, barley has gained attention in recent years due to the health claim approved by Health Canada in 2012, that says the consumption of barley beta-glucan has a blood cholesterol lowering effect (Government of Canada, Health Canada, Health Products and Food Branch Food Directorate, 2012). This led to increased interest among grain scientists to develop and evaluate food-grade barley varieties; that is, barley without a hull, and promote its production for improved human health and the Canadian agri-food industry. Currently, the production of hulless barley is less than the production of feed-grade barley and varieties for malting. In addition to the soluble fibre, beta-glucan, barley contains insoluble fibre including arabinoxylans. Barley is a source of energy (mainly in the form of starch), as well as vitamins, minerals and phytochemicals which are concentrated in the germ, aleurone and bran layers of the grain.

Some of the health benefits of cereal grains have been attributed to the presence of phytochemicals and micronutrients. Polyphenolic phytochemicals are the most abundant phytochemicals in plants. They include nutritionally important compounds such as flavonoids, polyphenols and phenolic acids. Polyphenols are divided into two categories: hydroxybenzoic acids and hydroxycinnamic acids. The hydroxybenzoic acids include gallic, *p*-hydroxybenzoic, vanillic, syringic, and protocatechuic acids. The hydroxycinnamic acids include *p*-coumaric,

caffeic, sinapic, ferulic, rosmarinic and chlorogenic acids. The hydroxycinnamic acids are derived from cinnamic acid through the shikimate pathway. Among the phenolic compounds found in grains, ferulic acid is the most abundant. All cereals contain phenolic acids in free and/or bound form (Dykes, L., & Rooney, 2007). Bound phenolics are often linked to carbohydrates in the cell wall via an ester bond. The majority of phenolic compounds in grains are located in the pericarp and aleurone layer. For example, among 11 varieties of barley, the greatest concentration of ferulic acid was found in the outer grain fraction, accounting for 77.7 to 82% of the total amount of ferulic acid in the grain. The outer layer also contained 79.2 to 86.8% of the ferulic acid dehydrodimers, and 78.0 to 86.3% of the *p*-coumaric acid content in the grain. Ferulic acid content was the most abundant hydroxycinnamate in the barley grains, with concentrations ranging from 359 to 624 µg/g dry weight, followed by *p*-coumaric acid (79-260 µg/g dry weight) (Hernanz et al., 2001).

The bioaccessibility and digestion of polyphenolic phytochemicals, such as ferulic acid, in cereal grain-based foods is of interest due to their antioxidant capacities. Ferulic acid is a well-known antioxidant. However, ferulic acid is commonly found crosslinked arabinoxylans, and this linkage limits the bioaccessibility of ferulic acid in the small intestine and alters its antioxidant capacity (Mateo Anson, van den Berg, Havenaar, Bast, & Haenen, 2009). *Bioaccessibility*, defined as the amount of ingested nutrient which is potentially available for absorption, is solely dependent on the release from the food matrix (Etcheverry, Grusak, & Fleige, 2012). The term *bioactivity* relates to the effect of a nutrient, and includes tissue uptake, physiological response, distribution/transport and metabolism (Fernández-García, Carvajal-Lérida, & Pérez-Gálvez, 2009). *Bioavailability* encompasses bioaccessibility, as it is comprised of both bioaccessibility and bioactivity. Bioavailability is defined as the amount of ingested nutrient that is absorbed and

available for physiological functions. Bioavailability is dependent on digestion, release from the food matrix, absorption by intestinal cells, and transport to cells in the rest of the body (Etcheverry et al., 2012). Bioavailability cannot completely be determined using *in vitro* methods, however, knowledge of the bioaccessibility of a particular phytochemical will help elucidate the *in vivo* bioavailability. Bioaccessibility can be measured *in vitro* by three methods: solubility, dialyzability, and gastrointestinal (GI) models such as the TIM system (TNO's Intestinal Model) which closely mimics dynamic *in vivo* digestion in humans. Simulated digestion includes lingual digestion by α-amylase in the mouth, gastric digestion with pepsin in the stomach, followed by pancreatic digestion with pancreatic amylase, lipase, ribonuclease, and proteases. Digestion is mimicked through the use of these enzymes, along with appropriately controlled conditions such as pH and temperature.

Although the antioxidant capacity of grain polyphenols has been studied extensively, their ability to exhibit antioxidant activity *in vivo* is poorly understood. In order to act as an antioxidant, phenolic acids must be released from the grain cell wall carbohydrates. Thus it is important to first know the bioaccessibility of specific polyphenols. In addition to cell wall carbohydrates, phenolic acids may interact with starch and proteins in the grain matrix. Therefore, the bioaccessibility of phenolic acids is influenced by several components within the grain matrix and there is a need for further research on its bioaccessibility in complex cereal grain matrices. Because ferulic acid is the predominant phytochemical in barley, ferulic acid was the focus of this research. Whole grain hulless barley varieties were examined due to the increased interest in barley consumption for beta-glucan, with the purpose of investigating the additional health benefits from its health-promoting phenolic content.

1.1. Hypotheses

1. The food matrix of cereals reduces the bioaccessibility of ferulic acid through its interaction with carbohydrates in the plant cell wall via an ester bond. Boiling and digestion of barley will lead to the breakdown/degradation of ferulic acid, resulting in a low bioaccessibility.

2. The level of free and bound ferulic acid changes throughout digestion based on its stability at each stage and is influenced by interactions with other components in the food matrix.

3. The thickness of the bran layer affects ferulic acid bioaccessibility; the thicker the bran layer, the lower the bioaccessibility. Additionally, barley varieties with a thicker bran will contain more ferulic acid.

4. The structure (degree of substitution (A/X ratio)) of arabinoxylans present in the barley grain may influence ferulic acid bioaccessibility. Total arabinoxylan content is positively correlated with bound ferulic and *p*-coumaric acid content. Furthermore, ferulic acid and arabinoxylan content, as well as the A/X ratio of the arabinoxylans differs among pearled barley fractions.

1.2. Objectives

Therefore, the main overall objective of this research was to determine the bioaccessibility of ferulic acid in hulless barley varieties and identify and characterize factors which may affect the bioaccessibility.

Specific objectives included:

1. Characterization of the phenolic acid composition before and after boiling, to identify the impact of processing

2. Simulation of gastrointestinal digestion and determination of the bioaccessibility of ferulic acid in barley by measuring the ferulic acid content in raw, boiled, and digested barley

3. Measurement of the amount of bioaccessible (free) ferulic acid at different timepoints of digestion

4. Measurement of the bran layer thickness and examination of its relationship with the release of ferulic acid, measured as free ferulic acid content

5. Characterization of pearled barley fractions in terms of ferulic acid and *p*-coumaric acid content, total AX, and A/X ratio, and establishment of potential correlation with the bioaccessibilities determined previously.

1.3. Rationale

Understanding bioaccessibility of ferulic acid is essential for examining the role of ferulic acid and possibly other phenolics in disease prevention, and paramount in identifying beneficial health effects of consuming cereal grains. More information is needed on the bioavailability and metabolic fate of polyphenols *in vivo*. Understanding the bioaccessibility will help determine the bioavailability of ferulic acid. Additionally, the detailed characterization of hulless barley in terms of phenolic content and their distribution in the grains is of interest to the food industry. The food industry has responded to the increase in consumer demand for healthier foods through the development of functional food products. Cereal grains rich in bioactive compounds are a good candidate for such product development. My research provides the necessary information to select hulless barley varieties or pearled barley fractions based on their level of bioactives for potential functional food development. These functional foods with an increased amount of bioaccessible ferulic acid may provide more antioxidant and anti-inflammatory effects.

CHAPTER 2.

Literature Review

2. Literature Review

2.1. Phenolic compounds in barley

Barley contains phenolic compounds including flavan-3-ols and phenolic acids. In one study (Mosele, Motilva, & Ludwig, 2018), flavan-3-ols accounted for the majority of free phenolic compounds in barley flour and primarily existed as dimers (procyanidin B3, prodelphinidin B3 and prodelphinidin B4) with procyanidin B3 and prodelphinidin B3 being the main dimers in barley (Holtekjølen, Kinitz, & Knutsen, 2006) . The identified flavanols in barley has been confirmed by Gangopadhyay, Rai, Brunton, Gallagher, & Hossain, (2016) using a different isolation and identification method. Other flavanols detected in barley include the trimer procyanidin C2, and monomers gallocatechin, catechin, catechinglucoside and catechindiglucoside (Mosele 2018). Procyanidins and catechin are found in the free soluble phenolic extracts of barley (Yoshida et al., 2010). Although flavanols are the major free phenolic compounds, phenolic acids are the major phenolic compounds overall in barley when accounting for the bound forms.

Phenolic acids in barley and other cereal grains are found predominately in the bound form, connected by ester or ether linkages to cell wall carbohydrates. Bound phenolic acids commonly found in barley include ferulic acid, diferulic acid, triferulic acid, *p*-coumaric acid, and vanillic acid. However, minor bound phenolic acids such as hydroxybenzoic acid, isoferulic, hydroxybenzoic, 2,4-dihydroxybenzoic, caffeic, sinapic and cinnamic acids have also been reported in small quantities (Martínez-Subirà et al., 2021; Yoshida et al., 2010). A diverse range of free phenolic acids have also been reported in barley including ferulic acid, vanillic acid, syringic acid, chlorogenic acid, protocatechuic acid, gallic acid, cinnamic acid, caffeic acid, *p*-hydroxybenzoic acid, benzoic acid and salicylic acid (Martínez-Subirà et al., 2021; Martínez et

al., 2018; Yang, Dang, & Fan, 2018; Yoshida et al., 2010; Yu, Vasanthan, & Temelli, 2001). Overall, the most reported phenolic acids in barley are caffeic, *p*-coumaric, and ferulic and diferulic acid, with ferulic and *p*-coumaric being the major ones. Other phenolic acids are identified in the literature but are not always quantified due to the very small amounts that cannot be measured accurately, as was the case in a study on phenolic acids in various barley varieties by Yu, Vasanthan, & Temelli, (2001). Although *p*-coumaric is among the most reported phenolic acids in barley, the isomers, *o*-coumaric acid and *m*-coumaric acid, are seldom reported and are less abundant in nature. One study could be found which reported *o*-coumaric acid in four hulless barley varieties cultivated in Tibet; *o*-coumaric acid was measured in the bound phenolic extracts of all four varieties, however, it was only measured in the free extract of the black-coloured variety (Jin, Dang, Zhang, Zheng, & Yang, 2022) . Future investigations on the phenolic acid composition of hulless barley may include measuring the *o*- and *m*- coumaric acid isomers in addition to *p*-coumaric, since genetics and the environment influence the phenolic acid composition.

It is well known that the most abundant phenolic acid in barley is ferulic acid. Ferulic acid accounts for 50-65% of the total phenolic acids found in barley, and more than 93% when considering bound phenolic acids only (Irakli, Lazaridou, Mylonas, & Biliaderis, 2020). Mosele et al (2018) reported a free ferulic acid content of 2.72 µmol/ 100 g dry weight and a bound ferulic acid content of 444.06 µmol/ 100 g dry weight. The ferulic acid content is highly variable. In a study conducted on 30 barley varieties including a combination of hulled/hulless/two-row/six-row/regular/waxy, the ferulic acid content ranged from 10.28 to 289.93 µg/g, depending on the variety as well as the extraction method (Yu et al., 2001). Although ferulic acid is most often identified as the main phenolic acid in barley, this study which used acid hydrolysis, α-

amylase cellulase hydrolysis treatments, identified *p*-hydroxybenzoic acid as the major phenolic acid among the 30 barley varieties studied (Yu et al 2001). Others who have examined ferulic acid in whole grain barleys reported values of 277.7 µg/g (Gangopadhyay et al., 2016), 246-485 µg/g (Hambira, 2009), 343-605 µg/g (Zupfer, Churchill, Rasmusson, & Fulcher, 1998), 555 to 663 µg/g (Du, Yu, Rossnagel, Christensen, & Mckinnon, 2009), 657-805 µg/g (Ndolo & Beta, 2014) and as much as 124-466 mg/g (Gamel & Abdel-Aal, 2012a). The diversity in phenolic acid composition and content among barley varieties has been described in several studies (Gamel & Abdel-Aal, 2012a; Hernanz et al., 2001; Irakli et al., 2020; Zupfer et al., 1998). Even studies which use similar extraction methods report different results, supporting the fact that ferulic acid content is cultivar dependent. Furthermore, environmental factors may play a role in ferulic acid content because the more stress on the plant such as in dry drought or cold conditions, the more phenolic acids are produced in response (Gamel & Abdel-Aal, 2012a). Additionally, variation is observed within the grain structure among botanical fractions, with the outermost layers containing the most ferulic acid. (Gamel & Abdel-Aal, 2012; Madhujith, Izydorczyk, & Shahidi, 2006;Ndolo & Beta, 2014).

Genotype as well as phenotype may influence ferulic acid content in grains. Smaller grains may contain more ferulic acid because of a higher percentage of aleurone and germ owing to a higher surface to volume ratio (Zupfer et al., 1998). More research is needed on the relationship between crease depth and ferulic acid content. However, it is known that there is a higher aleurone to pericarp ratio in the crease region. One study in wheat reported a lower amount of a ferulic acid dehydrotrimer in the crease region but high amounts of phenolic acids released by hot alkali treatment. Additionally, the authors reported that the feruloylated arabinoxylans contained ether linkages as opposed to the more common ester-linked compounds,

making this a unique feature of the crease region (Barron, Surget, & Rouau, 2007). Other physical characteristics which may influence ferulic acid content include the presence or absence of a hull, and whether the barley had a 2-row or 6-row structure. Regular (non-waxy) 2-rowed cultivars tend to contain less ferulic acid than 6-rowed cultivars (Yu et al., 2001; Zupfer et al., 1998). Another physical trait which may influence ferulic acid content is the thickness of the bran layer. The bran layer is rich in arabinoxylans which often have ferulic acid substituents. In fact, the arabinoxylan content of bran strongly correlates with ferulic acid content (Beaugrand, Crônier, Debeire, & Chabbert, 2004; Lempereur, Rouau, & Abecassis, 1997). Therefore, it is rational to hypothesize that bran thickness is also correlated with ferulic acid content, however, this has not been thoroughly studied. Only one study could be found reporting the bran layer thickness and ferulic content, and it found the contrary; the barley variety with the thickest bran layer had less bound ferulic acid than varieties with a thinner bran layer (Liu & Ng, 2016). Therefore, more studies are needed on the bran thickness and phenolic acid content of cereal grains. Bran thickness measurements have been obtained by fractionating the grain, conditioning the bran flakes to a moisture content of 17%, and measuring the thickness of individual bran flakes with a micrometer (Liu & Ng, 2016). However, the conditioning step causes swelling of the bran which results in a measurement twice as thick of that of the intact unmilled cut kernel. Although use of a micrometer is simple and cost effective, less destructive methods are needed in order to obtain accurate measurements of the bran layer as it exists in the whole kernel. Even the action of cutting the grain for imaging by scanning electron microscopy (SEM) alters the microstructure of the outer bran layers. Imaging technology typically used in medical research, such as MRI and CT, are non-destructive imaging techniques that have been minimally employed in plant research. Further application of this technology is needed on whole grain

barleys. Kernel weight does not seem to affect ferulic acid content. One study showed no relationship between kernel weight and ferulic acid content in barley and according to the authors, since kernel weight is strongly affected by environmental conditions, the lack of correlation suggests that phenolic acid content is primarily under genetic control (Zupfer et al., 1998).

2.2. Extraction of phenolic acids in barley

Phenolic acids can be extracted by various chemical, physical, and biological methods. By far the most commonly used method is the conventional solid-liquid chemical extraction on the grain flour which has been ground to a fine particle size and sieved. The choice of solvent should depend on the specific polyphenols of interest, plant material, level of conjugation and amount of hydroxyl groups in the structure; however, it is generally accepted that the extraction solvent should be highly polar since polyphenols are soluble in polar substances (Alara, Abdurahman, & Ukaegbu, 2021). For the extraction of free phenolic compounds from barley flour, ethanol, methanol or acetone is typically used, and following centrifugation, free phenolic acids are present in the supernatant. Ghafoor (2015) tested various conditions for the optimization of phenolic extraction from barley seeds including different temperature (45-60°C), time (3-6h) and ethanol concentration (65-80%). The authors concluded that the optimal condition deduced from the experiment for extraction of phenolic compounds from barley seeds was 65°C, 5.4 hour duration, and 65% ethanol concentration (Ghafoor, 2015).

The extraction of free phenolic compounds from a sample is typically done first, and then the remaining sample residue following free extraction is subjected to hydrolysis for extraction of bound phenolic compounds. These include compounds which are primarily located in the plant cell wall, bound to arabinoxylans and lignin, or physically trapped inside food matrices or cells

(Wang, Li, Ge, & Lin, 2020). To release them, the hydrolysis requires either strong acid (hydrochloric acid or sulfuric acid) or base (sodium hydroxide). Fewer studies use acid rather than alkaline hydrolysis due to the high temperature needed which can cause phenolic compounds to degrade (Bonoli, Verardo, Marconi, & Caboni, 2004). Additionally, boiling acid is hazardous and can cause glassware to break, splashing acid, and evaporation of water or other solvent leaving only acid which may result in burning of the samples. On the other hand, alkaline hydrolysis does not require heating during extraction. Additionally, alkaline hydrolysis leads to a higher recovery of phenolic acids from barley compared to acid hydrolysis (Verardo, Bonoli, Marconi, & Caboni, 2008). However, in one study, acid hydrolysis extracted a greater variety of phenolic acid compounds from barley compared to alkaline hydrolysis, in which the extracts only contained four main hydroxycinnamic acids namely two *p*-coumaric acid isomers and two ferulic acid isomers (Verardo et al., 2008). Despite the advantages of alkaline hydrolysis, acid hydrolysis was used to extract bound phenolic compounds from barley in some older studies (Bonoli et al., 2004; Verardo et al., 2008; Yu et al., 2001; Zupfer et al., 1998).

Non-conventional methods of phenolic extraction on barley and other cereal grains include physical methods such as ultrasound-assisted extraction (Madiha Yusoff, Mat Taher, Rahmat, & Suan Chua, 2022), as well as biological methods using enzymes such as alpha-amylase and cellulase (Yu et al., 2001; Yong Zhu et al., 2016). More recently, the focus is on efficient "green" methods which have a reduced impact on the environment since they use less chemical solvent and create less waste. The efficiency and potential valorization of green methods has been discussed in recent reviews (Ali Redha, 2021; Madiha Yusoff et al., 2022; Yeasmen & Orsat, 2021).

2.3. Antioxidant capacity of free and bound phenolic acid extracts in barley

Antioxidant compounds can donate a hydrogen atom or a single electron to neutralize harmful free radicals in the body or food matrix (Huang, Boxin, & Prior, 2005). There are several assays that can be used to measure the *in vitro* antioxidant activity of cereal grain extracts including 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC), trolox equivalence antioxidant capacity (TEAC), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ferric ion reducing antioxidant power (FRAP), and others (Huang et al., 2005). In barley studies, the most used assays are DPPH, TEAC and ORAC. Results on the antioxidant strength are based on trolox and are reported as trolox Equivalents (TE). Trolox is used since it is a water-soluble analog of vitamin E; therefore has similar antioxidant function as vitamin E but is more appropriate for the experimental assays due to its solubility in water.

Bonoli et al., (2004) compared the antioxidant capacity free and bound phenolic extracts of whole grain barley obtained by various extraction methods. The authors reported TEAC values ranging from 25.01 to 421 micromoles trolox equivalents per 100 g flour for the free extracts and 20.08 to 426.74 micromoles trolox equivalents per 100 g flour for the bound extracts. During processing, barley, like other cereal grains, is often subjecting to milling where the outer layers are removed. Pearling is a technique used in industry to remove the outer layers of the barley kernel. However, Madhujith et al., (2006) demonstrated the importance of keeping the outer layers during processing to avoid a substantial loss of bioactives. The study sequentially pearled barley into fractions and found that the level of phenolic acids and antioxidant activity (measured by TEAC, ORAC, DPPH, and photoinduced chemiluminescence technique) was greatest in the outermost layers and significantly decreased in fractions towards the endosperm. The TEAC

values among all fractions ranged from 0.45 to 59.71 micromole trolox equivalents per gram defatted material. TEAC values for the outermost layer were 81 to 131 times greater than that of the innermost fraction, depending on the variety (Madhujith et al., 2006). Correspondingly, phenolic acid content significantly decreased from the outermost fraction to the endosperm (Madhujith et al., 2006).

The positive correlation between phenolic compound content and antioxidant activity has been well established; however, it is important to note that there are also other antioxidant compounds in cereal grains in addition to phenolic acids. Water-extractable non-starch polysaccharides and feruloylated arabinoxylans from barley have also been found to have antioxidant activity (Malunga & Beta, 2015) and contribute to the antioxidant capacity of the whole grain. Furthermore, carotenoids present in the barley germ exhibit antioxidant activity; it was reported that the barley germ has a greater reducing capacity than that of the endosperm and aleurone fractions, due to the presence of carotenoids in the germ (Ndolo, Beta, & Fulcher, 2013). There is an abundance of research on the antioxidant capacity of wheat and wheat products; however, there are fewer studies on the antioxidant components in barley. Furthermore, there is a lack of information on the antioxidant capacity of barley once consumed. Simulated *in vitro* digestion experiments are needed to understand the beneficial health effects of barley phytochemicals.

2.4. *In vitro* digestion methods and models

Simulated digestion experiments aim to mimic the conditions of *in vivo* upper gastrointestinal digestion (Brodkorb et al., 2019) and can be used to investigate many different things. Although *in vivo* experiments are the ideal, they are costly and unethical in some circumstances. Simulated *in vitro* digestion methods have been demonstrated to correlate well with *in vivo* results, and the

advantages are that they are more feasible and less expensive. Digestion experiments are used to determine the impact of food structure and composition on human health, the digestibility or breakdown of nutrients such as proteins, carbohydrates and lipids, and measuring the release of micronutrients and phytochemicals from the food matrix (Minekus et al., 2014). Simulated *in vitro* digestion typically involves mimicking the conditions of oral, gastric and small intestinal digestion with physiologically relevant enzymes, pH and electrolytic digestive solutions. Some studies skip the oral phase altogether due to the short transit time (less than two minutes), and include only gastric and/or intestinal digestion. There is debate over whether the oral phase is necessary, and its inclusion may depend on the food under investigation. For example, studies involving the digestion of carbohydrate rich foods should consider including the oral phase since digestion of carbohydrates begins in the mouth where alpha-amylase in saliva starts to breakdown carbohydrates. During the gastric phase, the enzyme pepsin is added and the pH is reduced to acidic conditions (usually less than pH of 4) to mimic the conditions of the stomach. Gastric lipase is present *in vivo* but is currently not commercially available at this time for *in vitro* studies. During small intestinal digestion, either the enzyme cocktail pancreatin is added or the individual enzymes: porcine pancreatic alpha-amylase, porcine trypsin, bovine chymotrypsin, porcine pancreatic lipase, and porcine pancreatic colipase. The pH during this stage is increased to approximately 8. The enzyme concentration at each stage has varied among studies and in 2014, a prominent paper (Minekus et al., 2014) outlined the importance of standardizing digestion methods including recommended enzyme concentration at each stage. Building off of this work, in 2019 the COST Infogest Network, a team of interdisciplinary experts from around the world, published their recommendations for standardizing the digestion method (Brodkorb et al., 2019), with flexibility and variations depending on the research objectives and type of food

sample. The need for a standardized method was evident as the digestion methods used previously varied immensely among studies (e.g. various enzyme sources and concentrations, differing pH and electrolytic composition), making comparisons difficult. Terminating digestion and the treatment of samples post-digestion depends on the research objectives and desired analyses to be done on the digesta. Future studies employing this standardized method will facilitate comparison among results. However, there is a need for standardizing methods for specific food types and research objectives, to further facilitate appropriate comparisons among studies.

There are two main types of *in vitro* digestion models: static and dynamic. Static models are the simplest, most cost-effective method, and thus have been utilized the most in food research. They involve a constant pH at each stage of digestion and a constant ratio of food to enzymes (Brodkorb et al., 2019). However, static models lack the dynamic nature of digestion including gradual addition of enzymes and gastric fluid, pH gradient, and gradual stomach emptying (Brodkorb et al., 2019). Furthermore, there has been great variation in the conditions of static digestion used among researchers, although there are some methods that are most highly cited including those of Garrett, Failla, & Sarama, (1999) and Oomen et al., (2003), and more recently, Minekus et al., (2014) and Brodkorb et al., (2019). An example of a dynamic model is the TIM-1 system, which includes physiologically relevant dynamics such as changes in pH and enzyme concentration, and gradual gastric emptying. However, dynamic methods require complex maintenance, hardware and software and are overall more costly than static models.

The digestion conditions simulated usually represent the digestion which occurs in healthy adults. A standardized method is needed for simulating digestion for studies related to children and elderly. Moreover, much of the published methods use conditions which represent a fasting

state which is less relevant. The methods outlined by Infogest (Brodkorb et al., (2019) and Minekus et al., (2014) represent the fed state and future research should also simulate this state. Recent research on digestion models has focused on semi-dynamic models, which incorporate the advantages of both static and dynamic models but are more cost-effective than dynamic models. A semi-dynamic model was developed and used in a study on nutrient digestibility of dairy products by Mulet-Cabero, Rigby, Brodkorb, & Mackie, (2017) and in 2020, a recommended standardized protocol was published by the same group of international interdisciplinary experts (Mulet-Cabero et al., 2020).

Although the majority of digestion studies focus on macronutrient digestibility, there has been an increased interest in phytochemical and micronutrient bioaccessibility, which also requires simulated digestion experiments. For studies on the bioaccessibility of phytochemicals there are some additional considerations for the digestion method. The main challenges regarding the digestion experiment according to Brodkorb et al., (2019) are: "(i) the physiological appropriateness of the digestion conditions, such as reproducible matrix release and the sufficient presence of enzymes required for cleavage and cellular uptake, and (ii) separation of the bioaccessible phase from unavailable phytochemicals (e.g., precipitated or in complexed form), which can be achieved by centrifugation and/or filtration/dialysis." Another factor to consider for phenolic compounds is their susceptibility to light and oxygen. Digestion methods should limit the amount of oxygen and exposure to light to minimize degradation. This can be done by flushing the sample tubes with inert gas such as nitrogen (to remove oxygen), and covering the tubes to block light (Brodkorb et al., 2019). When measuring carotenoids, the shaking/mixing speed can enhance micellization and enhance bioaccessibility, and the amount of bile and pancreatin are other factors influencing carotenoid bioaccessibility (Brodkorb et al.,

2019). Therefore, these conditions must be carefully controlled when performing *in vitro* digestion for bioaccessibility studies on phytochemicals. To terminate digestion, enzyme activity is often stopped by heat shock (placing the sample tubes in 100 C for 5 min); however, this can breakdown the food structure and degrade phenolic compounds. Following digestion, for analysis of phytochemicals the samples should be centrifuged to collect the bioaccessible portion (supernatant). For analysis of polyphenols, it is suggested to pass the sample through a dialysis membrane to remove bound macromolecules (Bouayed, Deußer, Hoffmann, & Bohn, 2012; Brodkorb et al., 2019). If digestion timepoints are to be examined, it is suggested that separate sample tubes are terminated at various digestion stages as opposed to taking aliquots, due to the heterogeneity of digestion mixtures and the complexity of calculations required (Brodkorb et al., 2019).

2.5. Bioaccessibility of phenolic acids in barley

The bioaccessibility of a nutrient or bioactive describes the amount of the substance which is potentially available for absorption, and it primarily depends on its release from the food matrix (Etcheverry et al., 2012). Barley contains a greater amount of bound phenolic acids than free phenolic acids and only the portion of free phenolic acids which are present in the small intestine following digestion are considered bioaccessible. There has been a surge in phenolic acid bioaccessibility studies in cereal grains over the past decade; however, the majority of the work has been done on wheat and used *in vitro* methods (Gong, Chi, Zhang, Wang, & Sun, 2019; Hithamani & Srinivasan, 2014a; Tian et al., 2021; Zaupa et al., 2014). In wholewheat, the bioaccessibility of phenolic acids following *in vitro* gastrointestinal digestion was reported to be between 57.7% and 82.1% on average (Gong et al., 2019). There are fewer studies on the bioaccessibility of phenolic acids in barley. One *in vivo* study could be found on the

bioaccessibility of phenolic acids in grower pigs fed whole grain barley, and found that the bioaccessibility of total free phenolic acids was 83.23 % (Hole et al., 2013). Interestingly, the bioaccessibility increased to 85.68% when the pigs were fed extruded barley, indicating that extrusion impacts the content of free and bound phenolic acids and ultimately their bioaccessibility (Hole et al., 2013). In this study, the total tract bioaccessibility of individual phenolic acids differed: bound ferulic acid bioaccessibility was 61.97% compared to caffeic which had a bioaccessibility of 80.13% (Hole et al., 2013). In an *in vitro* study on hulless barley which examined total phenolic content, and antioxidant activity (DPPH, ABTS, and ORAC assays), the bioaccessibility of total or individual phenolic acids was not reported, however, the authors demonstrated that following *in vitro* digestion, the ABTS scavenging activities and ORAC values of the barley varieties were significantly increased. Since the total phenolic content and total flavonoid content did not correlate well with antioxidant values, the authors suggested that the antioxidant capacity observed may be attributed to the combined effect of phytochemicals, not solely phenolic or flavonoid compounds (Xia, Xing, & Kan, 2020). More research is needed on the level of specific phenolic and flavonoid compounds following digestion, which can be determined by chromatographic methods such as HPLC.

Only one other study could be found in the literature describing the bioaccessibility of phenolic acids in barley (Yulin Zhu, Yang, Huang, Huang, & Li, 2021). This *in vitro* study examined the total antioxidant capacity as well as bioaccessibility of phenolic acids in insoluble and soluble dietary fibres obtained from hulless barley following simulated gastrointestinal digestion. The authors reported the bioaccessibility of total phenolic content (TPC) rather than individual phenolic acids; however, the study does report the amount of each individual phenolic acid before and after digestion, leaving it up to the reader to calculate their individual

bioaccessibilities if desired. The bioaccessibility was calculated as the TPC at a given digestion stage divided by the initial TPC prior to digestion, expressed as a percentage. The bioaccessibility of total phenolic compounds increased following gastrointestinal (GI) digestion. The bioaccessibility of phenolic acids in the insoluble dietary fibre extracts was 96.29% after gastric digestion indicating that there were fewer phenolic compounds following gastric digestion compared to the undigested sample. However, after GI digestion, the bioaccessibility increased to 490.90%. In the soluble dietary fibre (SDF) extracts, the bioaccessibility also increased throughout digestion: 597.66% at the gastric stage and 1608.79% after GI digestion. The increase in phenolic content of SDF was primarily due to the increase in flavanol compounds following digestion and an increase in the hydroxybenzoic acid, gallic acid, while hydroxycinnamic acids such as ferulic acid and caffeic acid did not significantly increase throughout digestion. In the insoluble dietary fibre extracts, most of the identified phenolic acids decreased throughout digestion (Zhu et al., 2021). More research is needed on the bioaccessibility of ferulic acid in barley, especially food grade barleys for human consumption.

The different levels of individual phenolic acids detected following gastric and GI digestion indicate that the stability of these compounds differ. The stability of phenolic compounds may be related to their structure, and together, stability and structure influence their bioaccessibility (Dutra et al., 2017; Mosele, Macià, Romero, Motilva, & Rubió, 2015). More research is needed to fully understand what structural features are responsible for the stability of phenolic compounds during digestion and the role and effects the food matrix has on the stability and bioaccessibility of these compounds. Phenolic compounds that are released from the food matrix during digestion tend to have low stability (Gullon, Pintado, Fernández-López, Pérez-Álvarez, & Viuda-Martos, 2015), and therefore catabolized and not bioaccessible in the small

intestine. Understanding the susceptibility of each phenolic acid in gastric and intestinal environments will aid in an understanding of their bioaccessibility and the location in the GI tract where they exert their antioxidant effects. For example, the composition of the food matrix in carob flour has been shown to influence the stability and recovery of polyphenols following *in vitro* digestion. Specifically, the presence of soluble dietary fibre and added polyunsaturated fats (PUFAs) protected the polyphenolic compounds during duodenal digestion, enhancing their stability (Ortega, Macià, Romero, Reguant, & Motilva, 2011). This finding may explain the results of Zhu et al., (2021) in which the soluble dietary fibre fraction had a higher phenolic content following digestion, whereas insoluble dietary fibre phenolic content decreased. Research is needed on the impact of cereal grain matrices on the release of bound phenolic acids and the stability of free phenolic compounds in the GI environment.

Apart from whole grain barley studies, one study was found on the bioaccessibility of phenolic compounds in barley-flour-based products (Mosele et al., 2018). In addition to *in vitro* gastrointestinal digestion, this study also included colonic fermentation and is perhaps the only *in vitro* study on barley available in the literature currently, which includes digestion in the large intestine. After gastric digestion, the amount of total free phenolic compounds in the supernatant (bioaccessible fraction) relative to the starting material (undigested) was 16.3% in crackers, 11.9% in cookies, and 10.0% in pasta. Following intestinal digestion, the level of free phenolic compounds decreased further to 8.2%, 5.1% and 2.6% in cookies, crackers and pasta respectively. The level of free ferulic acid in the bioaccessible fraction also decreased throughout digestion of all products. Despite this similar downward trend throughout digestion, the bioaccessibility differed based on differing food matrices, for example, cookies had the greatest recovery of free phenolic compounds, and pasta had the lowest recovery, indicating that

processing and food matrix influence the digestibility of free phenolic compounds (Mosele et al., 2018). More research is warranted on understanding how the food composition and cooking influences the bioaccessibility of phenolic compounds in complex matrices.

2.6. Barley arabinoxylans

Arabinoxylans are non-cellulosic polysaccharides found in the cell walls of cereals and grasses (Izydorczyk, 2009). Arabinoxylans are the predominant compound within the non-starch polysaccharide component of cereal and grass cell walls. Arabinoxylans are present in various cereal grains including rye, wheat, barley, oats, rice, and sorghum (Izydorczyk & Biliaderis, 1995) with the highest content found in rye, followed by wheat (Izydorczyk, 2009). Cereal grain arabinoxylans are located in the cell walls of starchy endosperm, aleurone, bran, and sometimes the husk (Izydorczyk, 2009). In the lignified cell walls of pericarp tissue, arabinoxylans impart rigidity and strength by crosslinking to one another and to lignin. The secondary cell walls of cereals may have arabinoxylans referred to as arabino(glucorono)xylans. Arabino(glucorono)xylans have Ara*f* units as well as 2-O-linked α-D-glucopyranosyl uronic acid units and/or a 4-O-methyl derivative (Izydorczyk, 2009). Arabinoxylans are a form of dietary fibre and have the physiological effects of fibre and immunological effects as well. Furthermore, they have a technological impact in bakery products, as well as dairy products, film packaging, and pharmaceutical industry (Izydorczyk, 2009).

2.6.1. Structure and occurrence in whole grain and barley fractions

Arabinoxylans have a linear xylan backbone made up of β-(1→4) linked D-xylopyranosyl (Xyl*p*) units. Some Xyl*p* residues have α-L-arabinofuranose units attached as a side chain by α-1→3 and/or α-1→2 linkages at the *O*-2 and/or *O*-3 positions. Ferulic acid or *p*-coumaric acid may be attached to the arabinose units through ester bonds. The ferulate esters can dimerize

resulting in covalent crosslinking between arabinoxylan chains (Izydorczyk, 2009). This crosslinking impacts the solubility of arabinoxylans: the greater the amount of ferulate crosslinking, the lower the solubility of the arabinoxylan (Izydorczyk, 2009). This crosslinking also results in a more rigid and strong cell wall structure. The ferulate esters can also crosslink arabinoxylans to lignin and protein (Izydorczyk, 2009). Figure 2.1 shows the various arabinoxylan structural elements and linkages.

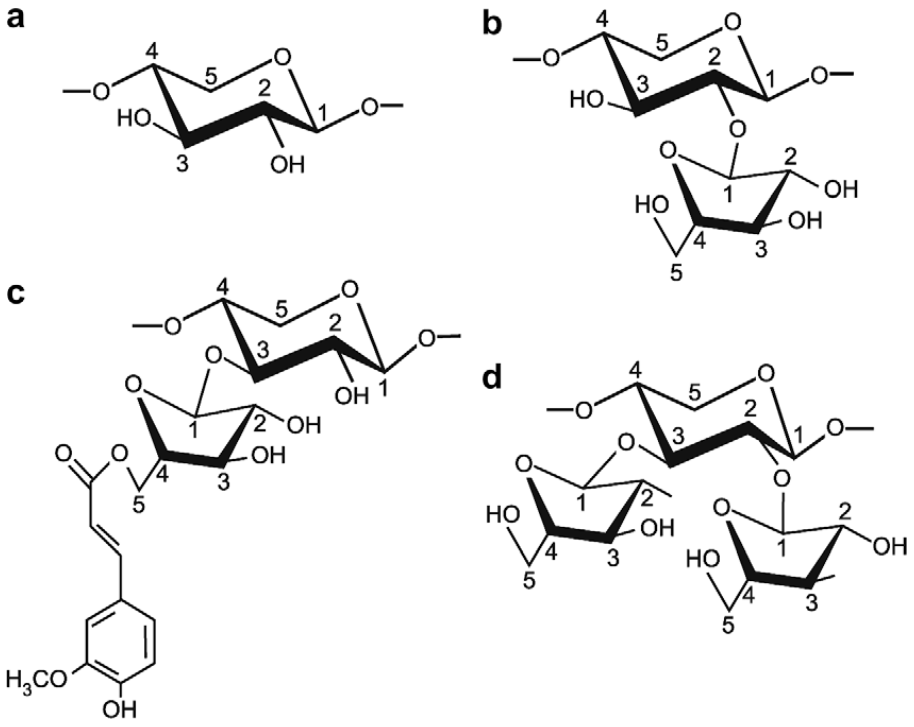

Figure 2.1. Schematic representation of **a)** unsubstituted Xyl*p*; **b)** monosubstituted Xyl*p* at O-2; **c)** monosubstituted Xyl*p* at O-3 with ferulic acid residue esterified to Ara*f* and **d)** disubstituted Xyl*p* at O-2,3 (From Izydorczyk & Dexter, (2008). Used with permission).

Arabinoxylans are diverse compounds, and can vary in substitution pattern, degree of substitution, monosaccharide composition, degree of polymerization of the xylan backbone, and in terms of the presence of attached constituents (Izydorczyk, 2009). The substitution pattern

describes the pattern of substitution with arabinose (mono- or di- substituted units). The degree of substitution is the extent to which the xylan backbone is substituted with arabinose units. It is often described as the ratio of arabinose to xylose (A/X) residues and indicates the degree of branching in the arabinoxylan structure. Therefore, the degree of substitution impacts the complexity of the arabinoxylan and potentially the bioaccessibility of attached residues such as ferulic acid. The monosaccharide composition of arabinoxylans describes the types and amounts of neutral sugars in the arabinoxylan. The degree of polymerization describes the length of the xylan backbone. Lastly, arabinoxylans can differ in the presence of attached constituents such as ferulic acid or *p*-coumaric acid.

For example, the monosaccharide content in wheat arabinoxylan varies slightly amongst wheat species, as demonstrated by Skendi, Biliaderis, Izydorczyk, Zervou, & Zoumpoulakis, (2011) when comparing six Greek wheat cultivars. The degree of substitution in wheat arabinoxylan ranges from 0.50-1.07 (Izydorczyk & Biliaderis, 1995). The variation is due to both genetic and environmental influences. Similarly, barley arabinoxylan content also depends on genetic and environmental factors. Marconi et al., (2020) demonstrated that the growing year significantly impacts the total arabinoxylan content in barley, as well as the amount of water extractable arabinoxylans (WEAX) and some structural characteristics of WEAX. Arabinoxylans can be categorized based on their structure and solubility: including WEAX, water-unextractable arabinoxylan (WUAX), alkali-extractable arabinoxylan (AEAX), and enzyme-solubilized arabinoxylan (ESAX).

Barley contains 5.8% arabinoxylans, which is similar to the arabinoxylan content in wheat (Izydorczyk & Dexter, 2008). However, this is lower than the amount in rye (7.6-12%), and more than oats (2.7-3.5%), rice (2.6%) and sorghum (1.8%) (Izydorczyk & Biliaderis, 2007). In

barley, the amount of arabinoxylans decreases from the outer layers towards the centre of the grain (Martínez-Subirà et al., 2021). Furthermore, the structural features of arabinoxylans may differ among botanical grain tissues, as they vary in durum wheat (Marcotuli et al., 2016) and extracted fractions (Storsley, Izydorczyk, You, Biliaderis, & Rossnagel, 2003). In barley, the structure of AEAX fraction has been described as highly branched β-(l→4)-xylan, including un-substituted (1,4-linked β-Xylp, 56.9 %), mono-substituted (1,2/3,4-linked β-Xylp, 22.1 %) and di-substituted (1,2,3,4-linked β-Xylp, 18.4 %) xylose units via (1→4) linkages (Li et al., 2020). The endosperm cell wall of barley has been described as having a bi-layered structure, with the outer cell wall consisting of arabinoxylan and pectin, and the inner wall consisting of beta glucan (Langenaeken et al., 2020).

2.6.2. Health benefits of barley arabinoxylans

In terms of health, arabinoxylans are a source of soluble dietary fibre and contribute very little energy. They pass through the gastrointestinal tract undigested and make it to the large bowel, where they act as prebiotics by feeding the bowel microbiota, where they may enhance the growth of beneficial bacteria and hinder growth of negative bacteria. Studies have shown that arabinoxylans may slow glucose uptake, lower glycemic response, and aid in weight management (Garcia et al., 2007). Since hydroxycinnamic acids such as ferulic acid are attached to arabinoxylans, arabinoxylans have been shown to provide antioxidant activity and protection from free radicals (Herrera-Balandrano et al., 2019; Malunga & Beta, 2015). Crosslinked arabinoxylans may even lower cholesterol levels (Bagdi, Tömösközi, & Nyström, 2017).

In breadmaking, arabinoxylans can act as a flour replacer. This has economic and health benefits, as it has been shown that a small amount of arabinoxylan can replace a relatively large amount of flour, while at the same time provide positive bread qualities (Koegelenberg &

Chimphango, 2017). Reducing the amount of flour reduces the glycemic index. In 2011, the European Food Safety Authority (EFSA) Panel of Dietetic Products, Nutrition, and Allergies (NDA) stated that there is a cause-and-effect relationship between consumption of wheat endosperm arabinoxylans and reduced post-prandial glycemic response. Furthermore, the panel concluded that eight grams of 'arabinoxylan-rich fibre' from wheat endosperm per 100g available carbohydrates is needed in order for individuals to obtain the claimed effect (EFSA Panel on Dietetic Products, 2011). Due to the demonstrated health benefits, more research is needed on identifying barley varieties with increased levels of arabinoxylans, including their distribution in the grain and characterization.

2.7. Summary of research gaps identified

The review of the literature on the bioaccessibility of phenolic acids in barley revealed some research gaps. Firstly, there is a need for more studies on the phenolic composition, arabinoxylan content and antioxidant capacity of food grade, hulless, varieties, since these are the varieties, consumers prepare at home. There is a need for *in vitro* digestion studies on barley. These studies are needed to understand the bioaccessibility and potential health effects of barley phytochemicals. Furthermore, inconsistent findings on the stability of phenolic acids during digestion may be attributed to the differences in food matrices, highlighting the need for research on the role of the food matrix. Currently, it is unclear which specific grain characteristics, including structure and composition, influence the bioaccessibility of phenolic acids, especially ferulic acid. For example, ferulic acid is known to be primarily present in grains in bound to arabinoxylans and arabinoxylans are known to have various structures. Although ferulic acid and arabinoxylans are closely linked, it is unknown whether the amount or structural characteristics of arabinoxylans affect ferulic acid bioaccessibility in grains.

CHAPTER 3.

Bioaccessibility of Phenolic Acids in Canadian Hulless Barley Varieties

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3. Abstract

Barley contains phenolic acids which are partially responsible for the health benefits of whole grains. The bioaccessibility of phenolic acids has been examined in wheat, millet, oats, and other grains; however, there are no studies on the bioaccessibility of phenolic acids in food-grade barley. Therefore, the objective of this study was to measure the bioaccessibility of phenolic acids in four cooked whole grain, hulless, barley varieties. An *in vitro* digestion model was used to mimic human upper gastrointestinal digestion. Boiling increased the extractability of bound phenolic acids. Digestion increased the level of free phenolic acids, indicating these phenolic acids are highly bioaccessible, and may be due to the release of bound phenolic acids during cooking and digestion. The major bioaccessible phenolic acids were ferulic and *p*-coumaric acid with their bioaccessibility ranging from 131-173% and 51-135%, respectively. Peru-35 had significantly greater bioaccessibility of ferulic acid compared to the other three varieties. A hydroxycinnamic acid amide not reported in boiled barley before, N^1, N^8- dicaffeoyl spermidine, was identified in the free phenolic extracts with a relatively high abundance compared to the phenolic acids and may provide additional anti-inflammatory and antioxidant functions. These cooked whole grain, hulless, barley varieties are sources of bioaccessible phenolic acids.

3.1. Introduction

Whole grain cereals are well known to have many health benefits including blood glucose management, and reduced risk of obesity and cardiovascular diseases (Cho et al., 2013). Contributing to the benefits of whole grains are phytochemicals, including phenolic acids which have antioxidant and anti-inflammatory properties (Beta & Camire, 2019; Jacobs, Andersen, & Blomhoff, 2007; Liu, Qiu, & Beta, 2010). The phenolic acids commonly found in whole grain cereals are *p*-hydroxybenzoic, protocatechuic, vanillic, gallic, syringic, caffeic, *p*-coumaric, ferulic and sinapic acids (Dykes, & Rooney, 2007). The amount of each phenolic acid varies from grain type and grain fraction. Most of the phenolic acids are covalently linked to the cell wall in the outer layers of the grain. Therefore, whole grains contain more phenolic acids compared to refined grains.

Processing technologies such as mechanical treatment, thermal treatment, extrusion cooking and bioprocessing have been used to liberate the phenolic acids from the outer grain layers and enhance their bioavailability (Wang, He, & Chen, 2014). Heat treatment can have an increasing or decreasing effect on the level of bioactives (Ruiz-Rodriguez, Marín, Ocaña, & Soler-Rivas, 2008), and the effect may depend on the grain type, grain matrix, and thermal processing conditions (Wang et al., 2014). Heat treatment may increase the level of extractable bioactives by loosening or releasing bioactives from the cereal matrices through thermal energy movement and the breakage of bonds in the cell wall (Wang et al., 2014). On the other hand, heat treatment may decrease the level of extractable bioactives due to the decomposition of heat-labile phenolic compounds (Wang et al., 2014). In extrusion cooking, a decrease in the level of extractable bioactives may be due to the polymerization of phenolics and tannins under the high heat and pressure conditions (Dlamini, Taylor, & Rooney, 2007).

Barley, *Hordeum vulgare*, is a nutrient dense cereal grain with components which make it a suitable crop for flour, beverage, and malt production. Barley varieties include those for animal feed, malt and food. Varieties for human consumption do not have a hull, i.e. are hulless, and are often sold as pearl or pot barley which have varying degrees of the outer grain layer removed to facilitate faster cooking. With the outer layers partially removed, these barleys have reduced phenolic acid content compared to the whole grain barley. Therefore, whole grain hulless barley varieties have potential to provide superior nutrition. However, for the phenolic acids to be bioavailable for utilization by the body, the phenolic acids must be bioaccessible, i.e. they must be released from the grain cell wall during digestion. The bonds between phenolic acids and cell wall constituents limits the bioaccessibility of the phenolic acids in the small intestine and alters its antioxidant capacity (Mateo Anson et al., 2009).

Bioaccessibility, defined as the amount of ingested nutrient which is potentially available for absorption, is solely dependent on the release from the food matrix (Etcheverry et al., 2012). The bioaccessibility of phenolic acids in the inner and outer regions of the aleurone layer varies, with the greatest variability among caffeic, *p*-coumaric, and sinapic acids (Zaupa et al., 2014). The bioaccessibility of a bioactive is primarily influenced by the food matrix and consequently, food processing which alters the matrix, influences bioaccessibility. The bioaccessibility of phenolic acids has been examined in wheat (Gong et al., 2019; Hithamani & Srinivasan, 2014a; Zaupa et al., 2014), millet (Hithamani & Srinivasan, 2014b), oats (Ryan & Thondre, 2012), and other grains (Hithamani & Srinivasan, 2014a), however, there are few studies on the bioaccessibility of phenolic acids in barley. No studies could be found on the bioaccessibility of phenolic acids in food-grade barleys. Therefore, the objective of this study was to measure the bioaccessibility of phenolic acids in cooked food-grade barleys. The extractability of phenolic acids following *in vitro*

digestion has been shown to be reduced, suggesting that phenolic acids are unstable in gastrointestinal conditions (Lang et al., 2020). Therefore, the authors hypothesized that the free phenolic acid content will be reduced following *in vitro* digestion, resulting in a low bioaccessibility.

3.2. Materials and Methods

3.2.1. Chemicals

Simulated digestive fluids were prepared using salts obtained from Fisher-Scientific Company (Ottawa, ON, Canada): potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), ammonium carbonate $(NH_4)_2CO_3$, calcium chloride ($CaCl_2$), Mallinckrodt Specialty Chemical Co. (Paris, Kentucky, USA): sodium chloride (NaCl), and Sigma-Aldrich Chemical Company (St. Louis, MO, USA): magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$). Bile extract porcine (B8631) was obtained from the Sigma-Aldrich Chemical Company along with the digestive enzymes: α-amylase from porcine pancreas 13U/mg (A3176), pepsin from porcine gastric mucosa ≥400U/mg protein (P7000), and pancreatin from porcine pancreas 8 X USP (P7545). To adjust the pH during digestion the following chemicals were used: pure HCl (37%) obtained from Acros Organics (Fair Lawn, NJ, USA) and $NaHCO_3$ from Fisher-Scientific Company. Solvents for phenolic extraction and HPLC analysis were HPLC grade methanol and ethyl acetate obtained from Fisher-Scientific and formic acid from Acros Organics. Folin-Ciocalteu reagent along with the phenolic acid standards (vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid) were purchased from Sigma-Aldrich Chemical Company.

3.2.2. Samples and cooking method

Four hulless varieties of barley grown between the years of 2012 and 2017 in Manitoba, Canada obtained from Agri-food and Agriculture Canada were examined. The varieties were Atahualpa, Peru-35, Roseland, and CI-1248. Atahualpa and Roseland were 2-row type and tan coloured. Peru-35 and CI-1248 were 6-row and black and purple coloured, respectively.

Barleys were boiled in the ratio of 1:3 (barley:water). Specifically, 50 g of barley and 150 ml distilled water was placed in a 600 ml beaker and covered with aluminum foil with venting holes. The barley and water were brought to a boil on a hotplate, and then the heat was reduced and allowed to simmer for 40 minutes. The barley absorbed the cooking water so all of the water was retained. The boiled samples were stored at -40 °C until ready for freeze drying. Following freeze drying, the cooked barley was ground to a coarse powder in a coffee grinder for 15 seconds and passed through a 0.5 mm mesh sieve.

3.2.3. *In vitro* digestion

The digestion procedure followed the standardized Infogest method outlined by Brodkorb et al., (2019) with modifications to the amount of bile used during intestinal digestion, and the enzymes used during gastric digestion.

3.2.4. Preparation of digestive fluids

Digestive electrolytic fluids including Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were prepared according to Brodkorb et al., (2019) with modification on the amount of bile extract in SIF. Solutions were stored at -20°C in aliquots and thawed as needed for each batch of digestion. The bile extract (Sigma-Aldrich Chemical Co, B8631) dissolved in SIF was prepared to result in 0.2 % w/v of the total digestive solution. Enzymes obtained from Sigma-Aldrich Chemical Co. included α-amylase (A3176), pepsin

(P7000), pancreatin (P7545, 8 X USP). Enzyme solutions were prepared fresh before each digestion. Solutions comprising 1500 U/ml of α-amylase in SSF,25000 U/ml pepsin in SFG, and 800 U/ml SIF were prepared.

Just prior to digestion, the enclosed shaking incubator was warmed up to 37°C. The digestive solutions were pre-warmed to 37°C.

3.2.5. Digestion

Two grams of the cooked, freeze-dried, and ground barley was hydrated with 1 ml Milli-Q water for each replicate. To the hydrated sample, 2.1 ml SSF was added, followed by 0.3 ml α-amylase (1500 U/ml in SSF), 15 µl 0.3 M calcium chloride, and 585 µl Milli-Q water to bring the total volume of 6 ml. The α-amylase concentration in the final oral solution was therefore 75 U/ml. The oral solution was mixed by vortexing and incubated at 37°C for 2 minutes.

To the 6 ml oral bolus, 4.5 ml SGF was added, followed by 960 µl pepsin (25000 U/ml in SGF), 3 µl calcium chloride, 400 µl water and 137 µl 1M HCl. The digestion mixture was vortexed, and the pH of the solution was verified to be 3.0. The mixture was incubated at 37°C for 2 hours under constant shaking. The activity of pepsin in the final mixture was 2000 U/ml.

To the 12 ml gastric bolus, 6.6 ml SIF was added followed by 3 ml pancreatin (800 U/ml) to achieve an activity of 100 U/ml in the final digestive solution. Next, 1.5 ml bile (0.38 g/ml in SIF) was added followed by 24 µl calcium chloride, 90 µl 1 M $NaHCO_3$ to adjust the pH to 7.0, and 786 µl Milli-Q water. The digesta was mixed and then incubated at 37°C for 2 hours. Digestion was terminated by placing the digestion tubes on ice and then storing them at -20°C until analysis.

3.2.6. Analysis Preparation

Samples were removed from the freezer and thawed at 4°C overnight. Digesta were transferred to conical flasks and centrifuged at 17090 x *g* for 15 min at 4°C. The supernatant (containing free

phenolics) and the pellet (containing bound phenolics) were collected into separate containers for freeze drying. The weight of each freeze-dried sample was measured. For raw and boiled samples, free phenolic extraction was conducted on the freeze-dried supernatant, and bound extraction was done on the freeze-dried pellet. For digested samples, only the supernatant was analyzed for free, bioaccessible, phenolic acids.

3.2.7. Free phenolic extraction

Free phenolic extraction was performed according to the method of Liu, Qiu, & Beta, (2010). For the gastric samples, 0.1 g of the freeze-dried supernatant was extracted upon. For the intestinal samples, 0.5 g of the freeze-dried supernatant was extracted upon. Free phenolics were extracted by adding 80% methanol to the freeze-dried samples followed by sonication for 1 hour. The samples were centrifuged at 7000 X g for 10 minutes at 10°C. The supernatant was collected into a clean tube and the residue was extracted again with 80% methanol following the same procedure. The supernatants were combined and then evaporated to dryness using a roto evaporator (100 rpm, 38°C). The dried extracts were reconstituted in 5 ml 50% HPLC grade methanol and then centrifuged at 18800 x *g* for 5 minutes. The supernatant was collected and filtered through a 0.22 µm filter into HPLC vials. Samples were stored at -20°C until HPLC analysis.

3.2.8. Bound phenolic extraction

Bound phenolic extraction was performed on the residue of freeze-dried raw and boiled barley samples. One gram of freeze-dried sample was hydrolyzed with 20 ml 4M NaOH, vortexed and left overnight at 4 °C. The samples were acidified to pH 1.5 with 6M HCl and then extracted 3 times with 35 ml ethyl acetate. The upper layer was collected each time and combined. The ethyl acetate extracts were dried to complete dryness using a roto-evaporator (100 rpm, 38 °C). The dried bound extracts were reconstituted in 2ml 50% HPLC grade methanol and centrifuged at

18800 x *g* for 5 minutes. The supernatants were filtered through a 0.22 µm filter into HPLC vials and stored at -20°C until analysis.

3.2.9. Total phenolic content (TPC) of free and bound extracts of raw and boiled barleys

TPC of free and bound extracts of raw and boiled barleys was determined using Folin-Ciocalteu reagent following the colorimetric method described by Ávila et al., (2019) with a few modifications, notably the volume of Folin-Ciocalteu reagent and incubation time. Ferulic acid was used as the standard for comparison. Samples were analyzed in triplicate in a 96 well plate. To each well, 240 µl distilled water followed by 12 µl sample, 12 µl Folin-Ciocalteu reagent, and 36 µl Na_2CO_3. The plate was incubated in darkness for 1 hour. Bound extracts required a further 5X dilution to give an absorbance reading within the range of the standard curve. On a separate well plate, a "standards plate" was similarly prepared which included 12 ferulic acid standards in sextuplicate, of the following concentrations: 0.8 mg/ml, 0.4 mg/ml, 0.2 mg/ml, 0.1 mg/ml, 0.05 mg/ml, 0.025 mg/ml, 0.0125 mg/ml, 0.00625 mg/ml, 0.003125 mg/ml, 0.0015625 mg/ml, 0.00078125 mg/ml, and 0.000399325 mg/ml. From these 12 standards, a standard curve of concentration versus average optical density (OD) was constructed and used to calculate the TPC of free and bound extracts of the barley samples. After the 1-hour incubation, plates were read at 750 nm using an Epoch microplate spectrophotometer (Synergy-BIOTEK, Winooski, VT, USA). The TPC was expressed as milligrams of ferulic acid equivalents per 100 g of barley on a dry weight basis.

3.2.10. HPLC analysis of raw, boiled, and digested barleys

Phenolic acids were identified and quantified using a Waters 2695 HPLC system equipped with a photodiode array detector (Waters 996) and a 100 x 3 mm reversed phase, C18 analytical column (Thermo Scientific, Accucore™ aQ Part no. 17326-103030). The following parameters

were used: column temperature = 35°C, sample temperature = 15°C, flow rate = 0.4 ml/min, and injection volume = 10 µl. Phenolic acids were separated with a gradient elution consisting of Mobile (A), water with 0.1% formic acid, and Mobile (B), methanol with 0.1% formic acid. The method was a 25- minute linear gradient elution programmed as followed: 0-3.81 min, 9-14% B; 3.81-4.85 min, 14-15% B; 4.85-5.89 min, 15-15% B, 5.89-8.32 min, 15-17% B; 8.32-9.71 min, 17-19% B; 9.71-10.40 min, 19-19% B; 10.40-12.48 min, 19-26% B; 12.48-13.17 min, 26-28 % B; 13.17-14.21 min, 28-35% B; 14.21-15.95 min, 35-40% B; 15.95-16.64 min, 40-48% B; 16.64-18.37 min, 48-53% B; 18.37-22.53 min, 53-70% B; 22.53-22.88 min, 70-9% B; 22.88-25.00 min, 9-9% B.

Phenolic acid standards including vanillic acid, ferulic acid, *p*-coumaric acid, caffeic acid, and sinapic acid, purchased from Sigma-Aldrich Chemical Co., were diluted to 5 different concentrations, ranging from 0.001563 mg/ml to 0.025 mg/ml, to generate a standard curve for each phenolic acid and used for comparison when quantifying the phenolic acids in the barley samples. Vanillic acid was measured at a wavelength of 280 nm and caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid were measured at 320 nm.

Further identification of a major phenolic compound in the samples was done by mass spectrometry on a Waters QTOF Micro Electrospray (Model, Manufacturer City, State, Country). The analysis was conducted in negative ion mode under the following conditions: nebulization gas- nitrogen, collision gas- argon, sample cone voltage- 20 V, capillary voltage- 1000 V, source temperature- 120 °C, desolvation temperature- 250 °C, and collision energies of 15, 30, and 45 eV for the compound of interest.

3.2.11. Statistics

The results were reported with the mean and standard deviation (SD) for triplicate analyses. The data was analyzed by a one-way analysis of variance (ANOVA) using SAS version 9.1 (SAS Institute Inc., Cary, NC) and significant differences were assessed among the raw, boiled and digested samples of all barley varieties, as well as the bioaccessibility of ferulic acid among the four varieties using Tukey's comparison with $p<0.05$.

3.3. Results and Discussion

3.3.1. Total phenolic content (TPC) of free and bound phenolic extracts

TPC of free and bound extracts of raw barley samples ranged from 853.71- 2331.93 µg FAE/g barley (dry-weight basis) (Figure 3.1). Boiling significantly increased the total bound phenolics and overall TPC in all barley types, except Peru-35 where the increase in bound phenolics was not statistically significant. The TPC of free and bound extracts of boiled barley samples ranged from 2177.49-2564.03 µg FAE/g barley (dry-weight basis). The greater amount of individual phenolic acids in the boiled extracts (Table 3.2) correlates with the greater TPC of boiled extracts compared to raw extracts (Figure 3.1). Boiling had no significant effect on the TPC of free phenolic extracts, except for in Atahualpa, where it significantly increased the TPC of the free extract. This finding agrees with the increase observed in individual free phenolic acids upon boiling. Therefore, the amount of bioaccessible phenolic compounds in whole grain barley was retained during cooking.

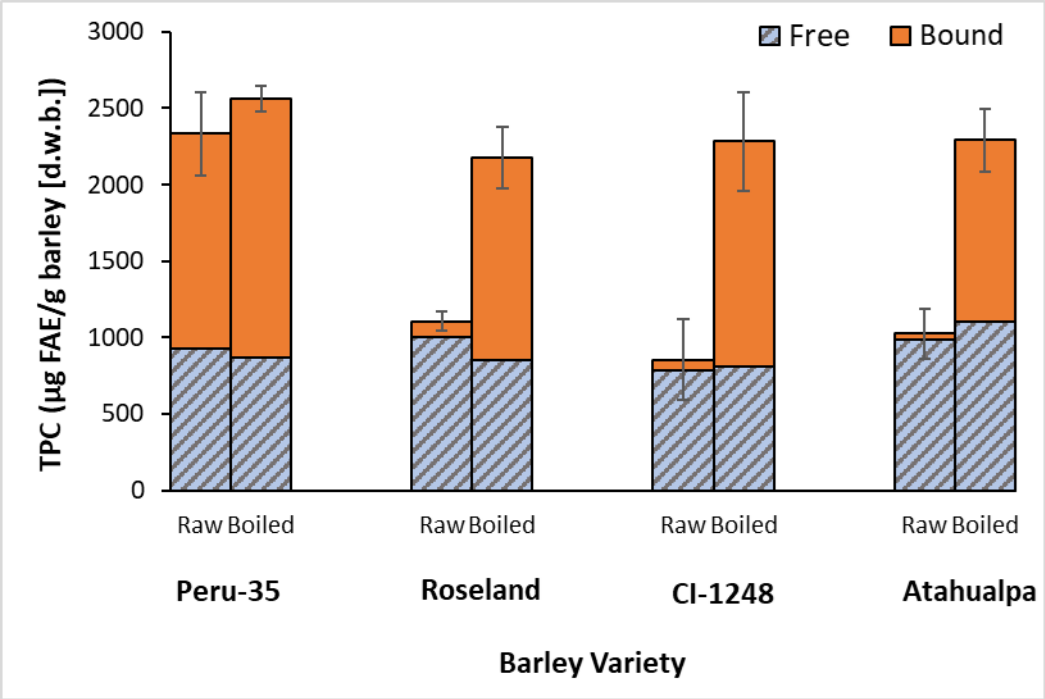

Figure 3.1. Total Phenolic Content (µg ferulic acid equivalent (FAE) per gram barley, dry-weight basis) of free and bound extracts for 4 types of hulless barley varieties, raw and boiled. Comparisons of TPC (free + bound extracts) of raw versus boiled samples for each barley type are indicated: bars with the same letter within a barley type are not significantly different ($P<0.05$). Error bars represent the standard deviation for the total phenolic content (free plus bound).

3.3.2. Identification and quantification of phenolic acids in free and bound phenolic extracts

HPLC facilitated the identification of phenolic acids in extracts of raw, boiled, and digested barleys with retention times compared to standards. Five types of phenolic acids were identified and quantified in the four barley varieties: ferulic acid (FA), *p*-coumaric acid (p-COA), caffeic acid (CFA), sinapic acid (SIA) and vanillic acid (VNA). Figure 3.2 shows the chromatograms of free and bound phenolic extracts of raw and boiled Peru-35.

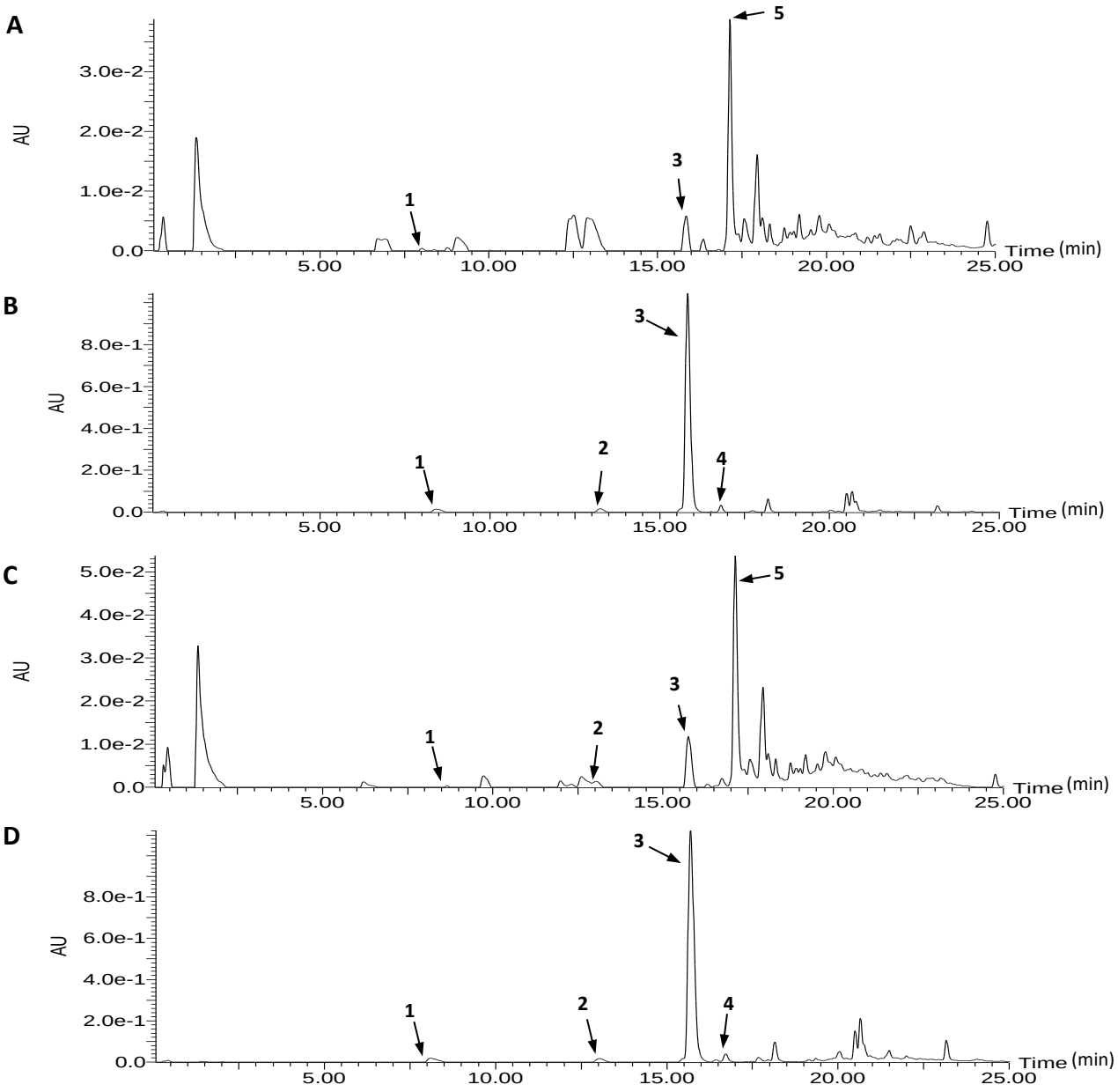

Figure 3.2. HPLC chromatograms at λ=320 nm showing the phenolic acid profiles of raw and boiled Peru-35. A: Raw free extract, B: Raw bound extract, C: Boiled free extract, D: Boiled bound extract. 1 – caffeic acid (RT~8.41 min), 2- *p*-coumaric (RT~13.25 min), 3- ferulic acid (RT~15.85), 4- sinapic acid (RT~16.82 min), and 5- N^1, N^8- Dicaffeoyl spermidine (RT~17.10 min).

The phenolic acid composition varied among Peru-35, Roseland, CI-1248 and Atahualpa, however, all four varieties contained *p*-coumaric acid and ferulic acid in the raw and boiled state. Ferulic acid was the dominant phenolic acid in the bound extracts, and the most abundant phenolic acid in the barley extracts overall (Table 3.2). Bound diferulic dimers were found in all four of the barleys, however, the bound monomeric ferulic acid was the dominant peak observed in the HPLC chromatogram (Figure 3.3). The bound ferulic acid monomer comprises of 50-62% of the total amount of phenolic acids in barley (Holtekjølen et al., 2006). This paper focused on the ferulic acid monomer rather than the dimers due to the greater abundance of the former in these samples. The composition and bioaccessibility of ferulic acid dimers in barley have been described elsewhere (Hernanz et al., 2001; Hole et al., 2013).

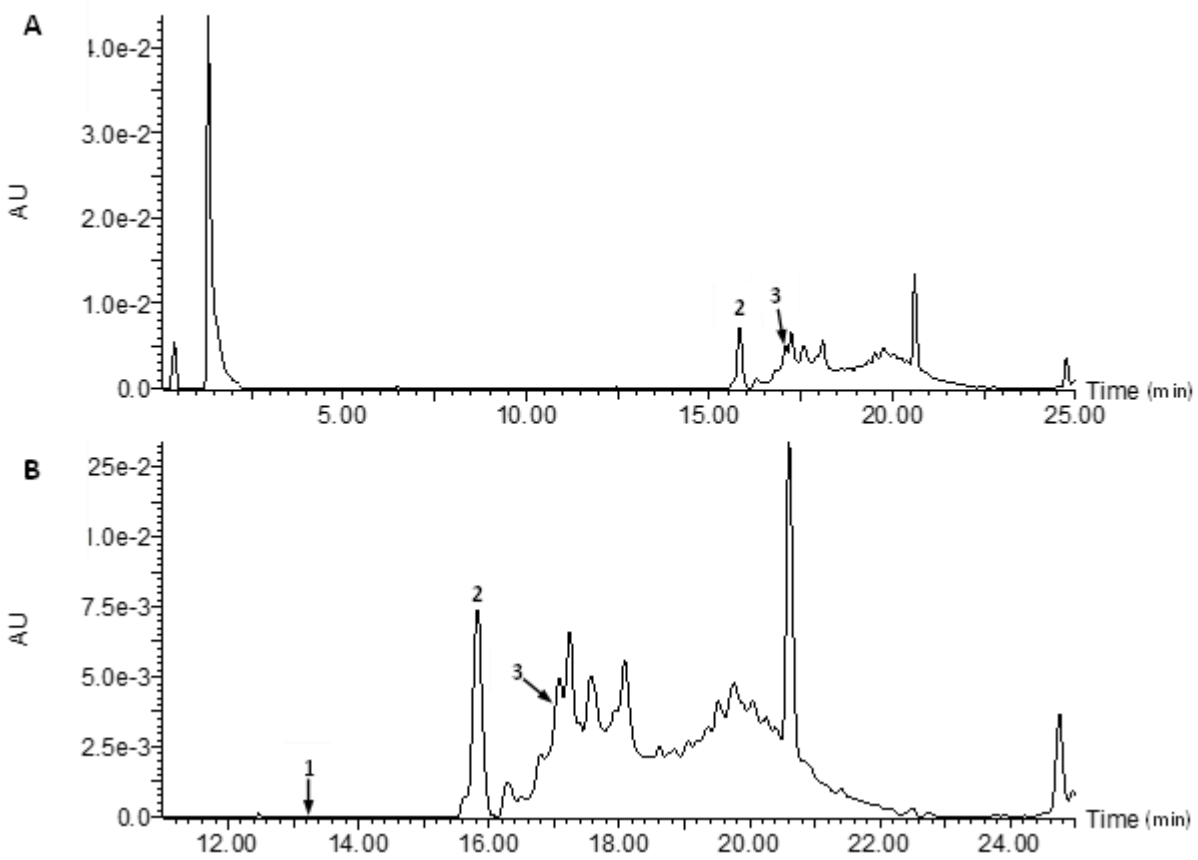

Figure 3.3. HPLC chromatogram of the free phenolic extract of digested Peru-35 at λ=320 nm, showing the bioaccessible phenolic acids: 1 – *p*-coumaric, and 2– ferulic acid, and 3– N^1, N^8- Dicaffeoyl spermidine. A– full chromatogram (25 minutes), B– zoomed in chromatogram of A showing 11.5 to 25 minutes. The tall peak at RT = 20.06 minutes is a background peak from the digestive solution, also found in the chromatograms of the control.

The phenolic acid profile of free extracts were more diverse than the bound extracts, containing ferulic acid, *p*-coumaric acid, caffeic acid, sinapic acid, vanillic acid, and diferulic

acids. These phenolic acids were found in the free extracts of all four varieties except for Atahualpa which did not contain free caffeic acid (Table 3.2). In terms of free phenolic acids, *p*-coumaric and ferulic were the most abundant phenolic acids in the free extracts after digestion. This finding suggests that the most bioaccessible phenolic acids in the whole grain barleys examined are ferulic acid and *p*-coumaric acid.

The types and amounts of phenolic acids present in barley depends on the barley variety, and the presence or absence of a hull (Holtekjølen et al., 2006). Vanillic acid is reported in certain barley varieties (Martínez et al., 2018; Yu et al., 2001). In this study, vanillic acid was only seen in the free extracts of raw Peru-35 and Roseland, free extracts of boiled CI-1248 and Atahualpa and digested free extracts of CI-1248 and Atahualpa. There was no vanillic acid in bound form, similar to the findings of Holtekjølen et al. (2006). Chlorogenic acid, protocatechuic, *p*-hydroxybenzoic acid have also been reported in barleys in acid hydrolysates and α-amylase hydrolysates (Yu et al., 2001), but were not found in this study. The varying results may be due to differences in the extraction method and barley variety.

Phenolic acid profiles were similar among raw and boiled barley samples, however, boiled samples contained significantly more phenolic acids than the raw barleys. Table 3.2 shows the amount of phenolic acids in free and bound phenolic extracts of the four barleys, raw and boiled. Some phenolic acids were found in the boiled barley samples which were not found in the raw flour. These results suggest that boiling increased the extractability of the phenolic acids, possibly through loosening of the food matrix, and breakage of the bonds between phenolic acids and the cell wall carbohydrates. Enhanced extractability of bound phenolic acids due to grain processing has been reported in other cereals including wheat, brown rice, and oats (Zeng, Liu, Luo, Chen, & Gong, 2016). The reason for enhanced extractability of phenolic

acids upon cooking is due to the thermal action of boiling which breaks down the structural components of cereal grains such as polysaccharides and proteins, as well as the leaching of phenolic acids contained in the cell vacuoles (Lang et al., 2020).

Table 3.1. Free and bound phenolic acid content (µg/g barley dry weight basis) in raw and boiled barley varieties: Peru-35, Roseland, CI-1248 and Atahualpa.

	Barley Variety	Peru-35		Roseland		CI-1248		Atahualpa	
		Raw	Boiled	Raw	Boiled	Raw	Boiled	Raw	Boiled
Vanillic acid	Free	4.54 ± 0.37	- †	7.70 ± 0.13	-	-	0.97 ± 1.04	-	7.32 ± 0.09
	Bound	-	-	-	-	-	-	-	-
Caffeic acid	Free	4.79 ± 0.68	4.85 ± 0.32	-	-	-	5.59 ± 0.93	-	-
	Bound	4.73 ± 0.25	6.07 ± 0.15	-	5.11 ± 0.35	-	8.65 ± 1.55	-	10.48 ± 1.61
***p*-Coumaric acid**	Free	-	6.08 ± 0.52	6.57 ± 0.19	6.45 ± 0.20	5.17 ± 0.66	6.58 ± 0.34	5.75 ± 0.17	7.23 ± 0.31
	Bound	3.94 ± 0.35	5.41 ± 0.12	1.67 ± 0.002	5.65 ± 1.25	-	4.09 ± 0.24	-	4.36 ± 0.37
Ferulic acid	Free	5.45 ± 0.33	7.94 ± 0.28	5.91 ± 0.27	12.58 ± 0.27	5.39 ± 0.47	8.16 ± 0.75	5.78 ± 0.6	8.005 ± 0.42
	Bound	102.53 ± 12.77	141.35 ± 7.33	11.35 ± 1.13	110.09 ± 5.17	9.69 ± 0.40	271.95 ± 20.92	7.06 ± 1.11	85.76 ± 7.83
Sinapic acid	Free	-	-	-	-	-	2.40 ± 0.16	5.86 ± 0.07	5.54 ± 0.12
	Bound	5.68 ± 0.39	7.74 ± 0.10	-	5.32 ± 0.31	-	5.39 ± 0.25	-	6.99 ± 0.43

† Note: (-) denotes not detected. The limit of detection (LOD) and limit of quantitation (LOQ) for each phenolic acid are as follows (µg/ml): vanillic acid LOD= 4.9, LOQ= 15.0; caffeic acid LOD= 1.6, LOQ= 4.8; *p*-coumaric acid LOD= 1.5, LOQ= 4.4; ferulic acid LOD= 1.5, LOQ= 4.4; and sinapic acid LOD= 1.5, LOQ= 4.5.

3.3.3. Identification of a hydroxycinnamic acid amide in the free phenolic extracts of barleys

In the free extracts of all four varieties there was a large peak (retention time (RT)=17.1 minutes) observed in the HPLC chromatogram which of the five phenolic standards (Figure 3.2). To investigate the identity of this compound, HPLC-MS/MS ESI negative ion mode was employed. Firstly, HPLC-MS was done to confirm the retention time for the unknown peak and to determine the mass in preparation for MS/MS. The compound had a m/z value of 468, indicating a mass of 469. HPLC-MS/MS yielded fragments with m/z values of 332, 306, 161, and 135 (Figure 3.4). This compound was tentatively identified as the hydroxycinnamic acid amide, *N*1, *N*8- Dicaffeoyl spermidine, which has also been reported in whole grain sorghum (Kang, Price, Ashton, Tapsell, & Johnson, 2016). The tentative identification of this compound in Peru-35, Roseland, Atahualpa, and CI-1248, was based on the HPLC-MS/MS data and by comparison with data in the literature. In sorghum, the HPLC-MS/MS (negative ionization mode) of the compound identified as N^1, N^8- Dicaffeoyl spermidine had fragments with m/z values of 332, 306, 276, and 161 (Kang et al., 2016), which is identical to the fragmentation pattern observed in this study. According to Kang et al., (2016), the base peaks of m/z 332, m/z 306 and m/z 161 indicate the presence of a caffeoyl group and amide moiety in the structure, which was further confirmed by MS^3 data.

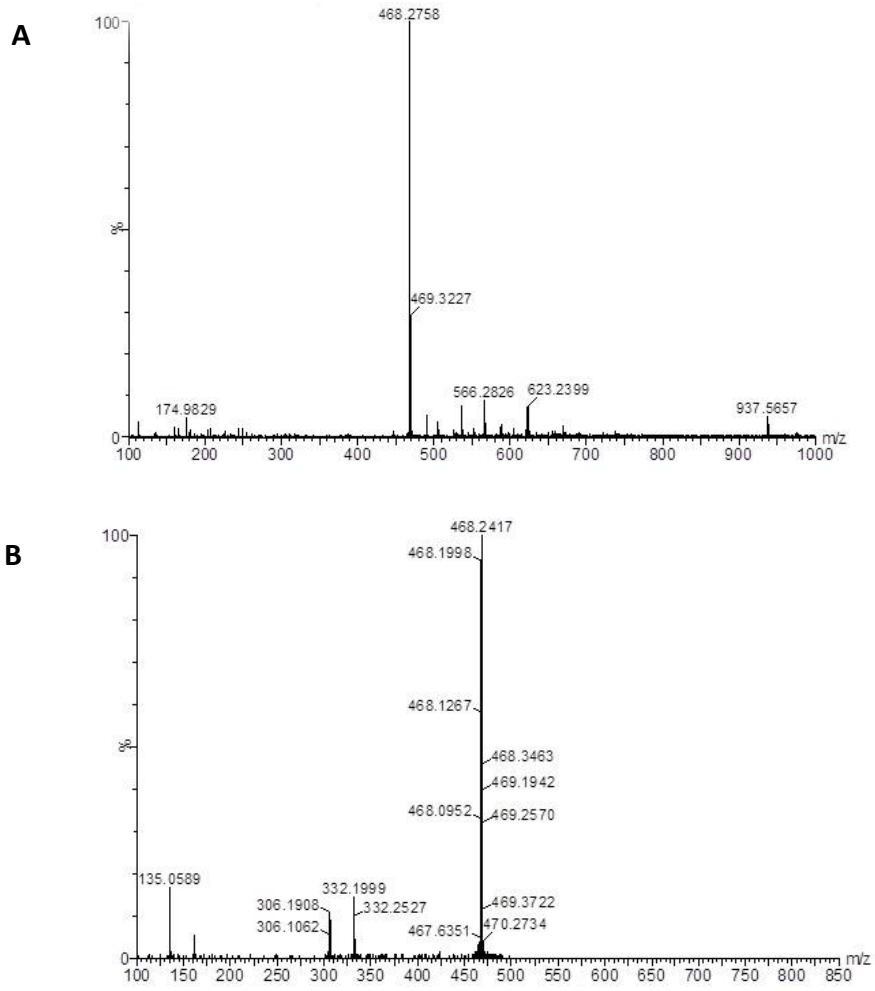

Figure 3.4. Representative mass spectra of the compound tentatively identified as N^1, N^8-Dicaffeoyl spermidine (A) and MS/MS (B) in the free phenolic barley extracts, obtained from Peru-35. The peak with had an m/z value of 468 $[M-H]^-$ and the MS/MS fragments had m/z values of 135, 306, and 332.

For the first time, a spermidine-containing compound, N^1, N^8- Dicaffeoyl spermidine has been reported in the free phenolic extracts of raw and cooked hulless, whole grain barley varieties: Peru-35, Roseland, CI-1248, and Atahualpa. N^1, N^8- Dicaffeoyl spermidine represented the largest peak observed in the HPLC chromatograms of free extracts (Figure 3.2). Although this compound was not quantified, it likely contributed to the TPC of the free extracts. From the TPC results of raw and boiled barley samples, it can be hypothesized that N^1, N^8- Dicaffeoyl spermidine is stable throughout boiling. The TPC of the free extract did not significantly change upon boiling (Figure 3.1), despite a significant increase in phenolic acids. This result may be explained by the relatively large contribution of N^1, N^8- Dicaffeoyl spermidine on the TPC of the free phenolic extracts. Furthermore, its presence in the free extract of digested samples indicates it is a bioaccessible compound in whole grain barley.

Hydroxycinnamic acid amides play a role in plant growth and in the response to biotic and abiotic stressors (Z. Li et al., 2018; Macoy, Kim, Lee, & Kim, 2015). Other hydroxycinnamic acid amides have been identified in barley leaves (Piasecka, Sawikowska, Krajewski, & Kachlicki, 2015). Polyamines such as spermidine have been reported in barley seedlings (Bird & Smith, 1984; Smith & Best, 1977), wheat flour and wheat germ, but not in whole grain barley. Polyamines may have an important role in the diet as they have anti-inflammatory and antioxidant properties (Madeo, Eisenberg, Pietrocola, & Kroemer, 2018), and spermidine has been shown to have cardioprotective effects and may extend lifespan (Eisenberg et al., 2016), as well as have neuroprotective effects (Gupta et al., 2013).

3.3.4. Bioaccessibility of phenolic acids in cooked barley

The bioaccessibility of phenolic acids in barley varied across variety and among the different phenolic acids. The bioaccessibilities shown in Table 3.2 were calculated as the amount of each phenolic acid (free form) in the digested samples divided by the amount in the boiled samples, expressed as a percentage. In barleys where the boiled samples contained a low free phenolic acid content, the respective digested sample contained minimal to none of the free acids. This was observed for caffeic acid in all four barley samples, as well as *p*-coumaric acid and sinapic acid in Atahualpa and CI-1248. In these samples, the concentration of the phenolic acids may have been too low in the final digest to be detected. It is also possible that the phenolic acids were transformed throughout digestion to other compounds either by hydrolysis or binding to other compounds. Phenolic acids can bind to macromolecules such as proteins and starch through hydrophobic and electrostatic interactions (Budryn et al., 2016) (Thuengtung & Ogawa, 2019). However, for vanillic acid, boiled Atahualpa and CI-1248 contained a low amount of vanillic acid (7.32 ± 0.09 and 0.97 ± 1.04, respectively), yet the digested samples contained a significantly similar level of vanillic acid. This suggests that vanillic acid may be less susceptible to degradation or conjugation with other compounds during digestion than *p*-coumaric and sinapic acid in Atahualpa and CI-1248. The food matrix of these barley types and the location of the free vanillic acid in the grain may also be responsible for the stability of vanillic acid throughout digestion. An increase in vanillic acid following *in vitro* digestion of quinoa sprouts has been reported previously, and was one of the three main phenolic acids present after digestion (Q. Zhang et al., 2020). In a study which examined the stability of 30 phenolic compounds throughout *in vitro* digestion including anthocyanins, flavanols, and phenolic acids, the stability of phenolic compounds varied and

phenolic compounds underwent different metabolic reactions (Hao et al., 2021). Further investigation into the stability of phenolic acids during digestion is warranted.

Contrarily, when the boiled samples contained a high level of free phenolic acid content, the digested sample contained more free phenolic acids than the boiled. This was observed for ferulic acid in all four barley varieties, and for *p*-coumaric acid in Peru-35. Thus, the bioaccessibility of the ferulic acid was over 100%, as was the bioaccessibility of *p*-coumaric acid in Peru-35. These findings reject our hypothesis that the extractability of free phenolic acids would be lower than that of boiled samples, resulting in a low bioaccessibility. Exceptions were observed for the bioaccessibility of *p*-coumaric acid in the Roseland variety (50.5%), and the bioaccessibility of vanillic acid in Atahualpa (84.9%). The high bioaccessibility observed in this study differs from values demonstrated by others, in which the bioaccessibility of ferulic acid in durum wheat aleurone fractions was low, less than 1% (Mateo Anson et al., 2009) and 32-51 % (Zaupa et al., 2014). In whole grain feed barley, the bioaccessibility of total free phenolic acids upon digestion in growing pigs was found to be 83.23% (Hole et al., 2013). Differences in the method of calculating bioaccessibility, food sample, and digestion model may be responsible for the differing results. However, the results of this study are in agreement with a study by Thuengtung, Niwat, Tamura, & Ogawa (2018), in which the level of phenolic compounds after *in vitro* digestion was greater than that in the raw grain. The authors suggested this is due to the breakdown of phenolic and protein interactions by digestive enzymes. Some phenolic acids may have bound to proteins during *in vitro* digestion, however, in this study, it appears the release of phenolic acids from the cell wall carbohydrates dominated, resulting in an increase in the content of free phenolic acids.

No other studies could be found on the bioaccessibility of phenolic acids in hulless barley. As the world continues to shift to whole grain consumption, there will be an increased demand for

hulless barley varieties. Boiling the barley grains in a similar fashion as rice, in which there is no residual water to drain off is the most common way to cook barley. The results show that all four hulless barley varieties, which were cooked in this manner, contained bioaccessible phenolic acids after boiling.

Since ferulic acid was the only phenolic acid which appeared in both the boiled and digested samples for all four barley varieties, only the bioaccessibility of ferulic acid was compared across the four varieties. The bioaccessibility of the other phenolic acids could not be compared among the varieties. The bioaccessibility of ferulic acid was significantly greater in Peru-35 than in Roseland, CI-1248, and Atahualpa (Table 3.2). The varieties contained similar levels of free ferulic acid in the raw and boiled state, however, following digestion Peru-35 had the greatest level of ferulic acid, resulting in a greater bioaccessibility. Further research on the effect of grain structure and composition on the release of ferulic acid is warranted.

The bioaccessibility of phenolic acids reported here may be greater than that of the bioaccessibility *in vivo*. In *in vivo* digestion, phenolic acids are modified by xenobiotic metabolizing enzymes in the upper gastro intestinal tract (Hole et al., 2013), so the amount of phenolic acids measured at the end of upper digestion may be lower than that in an *in vitro* model. In this experiment, only the predominant digestive enzymes, α-amylase, pepsin, and pancreatin, were employed in the *in vitro* digestion. Despite, the limitations, *in vitro* digestion has advantages over *in vivo* intervention trials including being easier to undertake and less expensive. Static digestion models show good correlation with *in vivo* results (Bohn et al., 2018; Sanchón et al., 2018) and are well suited for the analysis of phytochemical bioaccessibility (Brodkorb et al., 2019). The *in vitro* digestion method employed in this study followed the protocols developed by the

COST INFOGEST network outlined in Brodkorb et al. (2019), and facilitates the comparison of results with other studies which use this standardized method.

3.4. Conclusions

The *in vitro* bioaccessibility of phenolic acids was examined for the first time in food grade barley varieties. The major bioaccessible phenolic acids in cooked hulless barleys after *in vitro* digestion were ferulic acid and *p*-coumaric acid. The bioaccessibility of ferulic acid ranged from 131-173% and was found in the digest of all four barley varieties but was significantly higher in Peru-35. The bioaccessibility of *p*-coumaric acid was 51% in CI-1248 and 135% in Peru-35. The significantly greater amount of free phenolic acids in the digesta compared to the boiled barleys may be due to the release of bound phenolic acids during digestion. Further investigation of the changes that phenolic acids undergo throughout digestion is needed. A polyamine, N^1, N^8-Dicaffeoyl spermidine, was tentatively identified in the free phenolic extracts of the four barley varieties with a relatively high abundance compared to the phenolic acids and may impart additional anti-inflammatory and antioxidant functions. Cooked whole grain, hulless, barley is a source of bioaccessible phenolic acids.

3.5. Acknowledgements

This study was financed with the generous support of the Canada Research Chairs (CRC) Secretariat. Pamela Drawbridge acknowledges the financial support of the University of Manitoba Graduate Fellowship. The authors are grateful to Agriculture and Agri-Food Canada and the Brandon Research and Development Centre for donating barley samples. The authors are also grateful to Alison Ser for instrumental and technical assistance during LC-MS analysis, and May Lee, for assisting with sample preparation and laboratory experiments.

Connections between Chapters 3 and 4

The results reported in Chapter 3 provided new insights into the bioaccessibility of the major phenolic acids found in four hulless barley varieties. The results showed that ferulic and *p*-coumaric acids were the most abundant phenolic acids in the four barley varieties, and their bioaccessibilities differed among varieties, with Peru-35, having significantly greater ferulic acid bioaccessibility. The next chapter, Chapter 4, further investigates two of the four varieties examined in Chapter 3: Peru-35 and Roseland. These varieties had the greatest ferulic acid bioaccessibility determined in Chapter 3. Chapter four examines the bioaccessibility of ferulic acid throughout digestion to determine when the majority of this acid is released during the digestive process. The chapter also investigates factors which may affect the release of ferulic acid including digestive enzymes and pH.

CHAPTER 4.

Bioaccessibility of Ferulic Acid in Hulless Barley Varieties at Stages of Simulated *in vitro* Digestion

4. Abstract

Ferulic acid, the most abundant phenolic acid in plants, is known to have antioxidant, anti-inflammatory, and antimicrobial activity, among other potential physiological benefits. To provide the beneficial health effects *in vivo*, ferulic acid must be bioaccessible and bioavailable for absorption in the upper gastrointestinal tract. The majority of ferulic acid in barley is present in bound form in the bran layer, however, only the free form is bioaccessible. The objectives of this study were to investigate the bioaccessibility of ferulic acid at four different stages of simulated *in vitro* gastrointestinal digestion in two barley varieties, to determine when the majority of ferulic acid is released during digestion and investigate factors which may affect the release of ferulic acid including digestive enzymes and pH. Following simulated *in vitro* digestion, free and bound ferulic acid was chemically extracted and then quantified using HPLC. Additionally, the barleys were characterized in terms of bran layer thickness using micro computed tomography (microCT) imaging to determine if this structural grain characteristic plays a role in determining ferulic acid bioaccessibility. This is the first study to measure the bran thickness of barley using microCT imaging with preliminary findings indicating enhanced ferulic acid bioaccessibility in grains with a thinner bran layer. The results showed that the release of ferulic acid was strongly influenced by digestive enzymes and minimally affected by pH. The major release of bound ferulic acid in barley occurred during the final hour of simulated small intestinal digestion. The amount of free, bioaccessible ferulic acid remained constant throughout digestion.

4.1. Introduction

The consumption of whole grain cereals has been linked to blood glucose management and the prevention of obesity and cardiovascular diseases (Cho et al., 2013). Some of the beneficial effects may be attributed to the antioxidant properties of whole grains due to their phytochemical content, including phenolic compounds. Ferulic acid is the most abundant phenolic acid in cereal grains and is commonly found linked to the cell wall carbohydrates such as arabinoxylans in the outer layers of the whole grain kernel. In barley, the outer grain layers contain both ferulic and *p*-coumaric bound to arabinoxylans, with the former being predominant. The linkage of these phenolic acids makes them inaccessible during digestion. To provide antioxidant effects *in vivo*, the phenolic acids must be bioaccessible and bioavailable for absorption in the upper gastrointestinal tract.

Gastrointestinal digestion comprises of oral, gastric and small intestinal digestion. During the oral phase of digestion, the food bolus is wetted and masticated, while salivary α-amylase begins to break down starch. The bolus moves on to the stomach where the gastric phase begins and lasts for two to four hours, depending on the type of food consumed. In the stomach, electrolytic gastric juices are secreted which contain the enzyme pepsin are at pH 3. Pepsin breaks down proteins to amino acids. In the small intestine, lipid digestion occurs by lipase, and proteins and carbohydrates are further broken down by trypsin and pancreatic α-amylase respectively. Bile is secreted into the small intestine to assist with lipid digestion and the amount depends on the lipid content of the bolus. Brush border cells in the small intestine absorb the nutrients and bioactives such as free phenolic acids released during digestion, where they enter the blood stream and are metabolized by the body. The remaining undigested bolus moves to the large intestine and colon where

fermentation takes place before being excreted. Therefore, bound phenolic acids that are inaccessible in the small intestine, may elicit antioxidant effects in the large intestine or colon.

Barley is gaining importance as a food particularly with the development of hulless types. Barley products containing beta-glucan gained a health claim by Health Canada for having a blood cholesterol lowering effect (Government of Canada, Health Canada, Health Products and Food Branch Food Directorate, 2012). Beta-glucan is concentrated in the endosperm fraction of the grain (Irakli et al., 2020; Izydorczyk et al., 2014), however, there are other health-promoting bioactives in whole grain barley, including phenolic acids. In hulless whole grain barley, the majority of phenolic acids are located in the bran which consists of the pericarp layer and multiple layers of aleurone cells, depending on the variety. The grain matrix is known to influence the bioaccessibility of phenolic acids and other phytochemicals, however, it is unclear which factors such as grain characteristics most affect the release of phenolic acids. The bran layer thickness of barley may be an important factor. Bran contains various carbohydrates such as arabinoxylans and beta-glucan, as well as lignin and proteins. The complexity and packing of the matrix and thickness of the bran may hinder the accessibility of carbohydrates to digestive enzymes. Traditionally, bran layer thickness is measured using manually cut grains and microscopy. Computed tomography (CT) is traditionally used as a medical diagnostic tool however, it is also useful for studying plant morphology. CT is a non-invasive, non-destructive, 3D imaging technique based on differential x-ray attenuation due to differences in the material's composition and density (Hughes, 2019). Therefore, microCT is an excellent tool for examining grain size and shape. One study used microCT to measure barley grain characteristics such as kernel width and aleurone thickness (Hughes et al., 2019), however no study could be found on its bran layer thickness (pericarp plus aleurone layers).

Additionally, since ferulic acid is found linked to arabinoxylans, the structure of arabinoxylans in grains may influence the release of ferulic acid during digestion, ultimately determining the bioaccessibility. Previously, it was determined that the level of insoluble bound ferulic acid in cereal grains (maize and wheat) is correlated with the feruloylated arabinoxylan content in gastric digesta (Malunga and Beta, 2016). Arabinoxylans are carbohydrates found in the bran layer of barley and other cereal grains and provide strength to the cell wall structure by crosslinking to one another via ferulate bridges and to lignin. Therefore, the bran layer thickness may be an indirect measurement of the amount of arabinoxylans and consequently ferulic acid content as well.

An understanding of the bioaccessibility and fate of phenolic acids during digestion helps to comprehend the physiological impact and health benefits of a particular food. Bioaccessible free phenolic acids in the stomach may conjugate with glucuronic acid and then are absorbed by brush border cells in the small intestine, whereas bound phenolic acids continue to the large intestine where they are further metabolized and degraded by microbial esterases and bacteria (Tarko, Duda-Chodak, & Zajac, 2013). There is currently only one study, to the best of my knowledge, on the behaviour of phenolic acids in barley and barley products throughout digestion (Mosele et al., 2018).

The bioaccessibility of phytochemicals, especially carotenoids, has been examined in foodstuff previously using *in vitro* digestion methods and have shown good correlation with *in vivo* results (Brodkorb et al., 2019) . However, there are few studies on the bioaccessibility of phenolic acids in barley. In growing pigs, the bioaccessibility of total free phenolic acids in barley was 83.23% and increased by 10% following extrusion (Hole et al., 2013). Research on the bioaccessibility of phenolic acids in food-grade (hulless) barley varieties *in vitro* and *in vivo* is

needed. Since ferulic acid was previously found to be the most abundant and bioaccessible phenolic acid in these barley samples (Drawbridge et al., 2021, there is increased interest in understanding its fate throughout digestion.

Therefore, the aim of this research was to investigate the bioaccessibility of ferulic acid at four different stages of *in vitro* digestion: half-way through gastric, gastric, half-way through intestinal, and intestinal phases, in two different cooked barley varieties, to determine when the majority of ferulic acid is released during digestion. To investigate factors which may affect the release of ferulic acid, food stability and pH controls were used to determine the effect of enzymes and pH. The low pH of the gastric phase is known to break the arabinose linkages in arabinoxylans (Malunga & Beta, 2016; Zhang, Zhang, & Whistler, 2003). Additionally, the varieties were characterized in terms of bran layer thickness to determine if this structural grain characteristic plays a role in determining ferulic acid bioaccessibility.

4.2. Materials and Methods

4.2.1. Chemicals

Simulated digestive fluids were prepared using salts obtained from Fisher-Scientific Company (Ottawa, ON, Canada): potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), ammonium carbonate $(NH_4)_2CO_3$, calcium chloride ($CaCl_2$), Mallinckrodt Specialty Chemical Co. (Paris, Kentucky, USA): sodium chloride (NaCl), and Sigma-Aldrich Chemical Company (St. Louis, MO, USA): magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$). Porcine bile extract (B8631) was obtained from the Sigma-Aldrich Chemical Company along with the digestive enzymes: α-amylase from porcine pancreas 13U/mg (A3176), pepsin from porcine gastric mucosa ≥ 400U/mg protein (P7000), and pancreatin from porcine

pancreas 8 X USP (P7545). To adjust the pH during digestion the following chemicals were used: pure HCl (37%) obtained from Acros Organics (Fair Lawn, NJ, USA) and $NaHCO_3$ from Fisher-Scientific Company. Solvents for phenolic extraction and HPLC analysis were HPLC grade methanol and ethyl acetate obtained from Fisher-Scientific and formic acid from Acros Organics. Folin-Ciocalteu reagent along with the phenolic acid standards (vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid) were purchased from Sigma-Aldrich Chemical Company.

4.2.2. MicroCT imaging of grains to measure bran layer thickness

Intact raw whole grain barley kernels were imaged using a Skyscan 1176 microCT scanner (Bruker, Billerica, MA, USA) at The Small Animal and Materials Imaging Core Facility in the Rady Faculty of Health Sciences, University of Manitoba. Nine kernels of each barley variety were imaged, and an average bran layer thickness was determined from images of transverse, sagittal, and coronal slices using the software DataViewer (Bruker). The pixel size was 8.894 micron. Specifically, 10 measurements at specific locations were obtained from each of the three views (transverse, sagittal and coronal) for each kernel, for a total of 30 measurements per kernel. Three-dimensional viewing was done with the program CTvox (Bruker). When analyzing the 3D images, botanical features of the grain kernels were used as landmarks to locate different areas of the grain to measure the bran thickness and obtain an average thickness for the kernel, as well as provide consistency in the measurement locations among different grain kernels.

4.2.3. *In vitro* digestion

The Roseland and Peru-35 barley samples used here were the same samples that were used previously for Chapter 3. The preparation of these samples included boiling, freeze-drying, and grinding and is described in Chapter 3, section 3.2.2, “Samples and cooking method”. The cooked

barleys were digested based on the standardized Infogest method of Brodkorb et al., (2019), as modified by Drawbridge et al., (2021).

4.2.4. Preparation of digestive fluids

The digestive electrolytic fluids including Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were prepared according to the method of Drawbridge et al. (2021). Solutions were stored at -20°C in aliquots and thawed as needed for each batch of digestion. The bile extract (Sigma-Aldrich Chemical Co, B8631) dissolved in SIF was prepared to result in 0.2 % w/v of the total digestive solution. Enzymes obtained from Sigma-Aldrich Chemical Co. included α-amylase (A3176), pepsin (P7000), pancreatin (P7545, 8 X USP). Enzyme solutions were prepared fresh before each digestion. Solutions comprising 1500 U/ml of α-amylase in SSF,25000 U/ml pepsin in SFG, and 800 U/ml SIF were prepared.

Just prior to digestion, the enclosed shaking incubator was warmed up to 37°C. The digestive solutions were pre-warmed to 37°C. Two grams of each ground, freeze-dried barley replicate was hydrated with 1 ml Milli-Q water.

4.2.5. Digestion

To the hydrated sample, 2.1 ml SSF was added, followed by 0.3 ml α-amylase (1500 U/ml in SSF), 15 µl 0.3 M calcium chloride, and 585 µl Milli-Q water to bring the total volume to 6 ml. The α-amylase concentration in the final oral solution was therefore 75 U/ml. The oral solution was mixed by vortexing and then incubated at 37 °C for 2 minutes.

To the 6 ml oral bolus, 4.5 ml SGF was added, followed by 960 µl pepsin (25000 U/ml in SGF), 3 µl calcium chloride, 400 µl water and 137 µl 1M HCl. The digestion mixture was vortexed, and the pH of the solution was verified to be 3.0. The mixture was incubated at 37°C for 2 hours under constant shaking. The activity of pepsin in the final mixture was 2000 U/ml.

To the 12 ml gastric bolus, 6.6 ml SIF was added followed by 3 ml pancreatin (800 U/ml) to achieve an activity of 100 U/ml in the final digestive solution. Next, 1.5 ml bile (0.38 g/ml in SIF) was added followed by 24 µl calcium chloride, 90 µl 1 M $NaHCO_3$ to adjust the pH to 7.0, and 786 µl Milli-Q water. The digesta was mixed and then incubated at 37°C for 2 hours. Digestion was terminated by placing the digestion tubes on ice and then storing them at -20°C until analysis. The control contained everything: all the digestive fluids, enzymes, and acid/base, except no barley sample. The food stability control (FSC) contained everything except enzymes. The pH control had everything including enzymes, but there was no pH adjustment with acid and base. The FSC and pH controls were done at two timepoints: 1) at the end of the gastric phase, and 2) at the end of the intestinal phase. For reaction termination and analysis, the controls were treated similarly to the samples.

4.2.6. Analysis Preparation

Samples were removed from the freezer and thawed at 4°C overnight. Digesta were transferred to conical flasks and centrifuged at 17090 x *g* for 15 min at 4°C. The supernatant (containing free phenolics) and the pellet (containing bound phenolics) were collected into separate containers for freeze drying. The weight of each freeze-dried sample was measured. For raw and boiled samples, free phenolic extraction was conducted on the freeze-dried supernatant, and bound extraction was done on the freeze-dried pellet. For digested samples, only the supernatant was analyzed for free, bioaccessible, phenolic acids.

4.2.7. Free phenolic extraction

Free phenolic extraction was performed according to the method of Liu, Qiu, & Beta, (2010). For the gastric samples, 0.1 g of the freeze-dried supernatant was extracted upon. For the intestinal samples, 0.5 g of the freeze-dried supernatant was extracted upon. Free phenolics were extracted

by adding 80% methanol to the freeze-dried samples followed by sonication for 1 hour. The samples were centrifuged at 7000 x *g* for 10 minutes at 10°C. The supernatant was collected into a clean tube and the residue was extracted again with 80% methanol following the same procedure. The supernatants were combined and then evaporated to dryness using a rotary evaporator (100 rpm, 38°C). The dried extracts were reconstituted in 5 ml 50% HPLC grade methanol and then centrifuged at 18800 x *g* for 5 minutes. The supernatant was collected and filtered through a 0.22 µm filter into HPLC vials. Samples were stored at -20°C until HPLC analysis.

4.2.8. Bound phenolic extraction

Bound phenolic extraction was performed on the residue of freeze-dried boiled and freeze-dried digested barley samples. One gram of freeze-dried sample was hydrolyzed with 20 ml 4M NaOH, vortexed and left overnight at 4 °C. The samples were acidified to pH 1.5 with 6M HCl and then extracted 3 times with 35 ml ethyl acetate. The upper layer was collected each time and combined. The ethyl acetate extracts were dried to complete dryness using a rotary evaporator (100 rpm, 38 °C). The dried bound extracts were reconstituted in 2ml 50% HPLC grade methanol and centrifuged at 18800 x *g* for 5 minutes. The supernatants were filtered through a 0.22 µm filter into HPLC vials and stored at -20°C until analysis.

4.2.9. HPLC analysis

Phenolic acids were identified and quantified using a Waters 2695 HPLC system equipped with a photodiode array detector (Waters 2996) and a 100 × 3 mm reversed phase, C18 analytical column (Thermo Scientific, Accucore aQ Part no. 17326-103030). The following parameters were used: column temperature = 35 °C, sample temperature = 15 °C, flow rate = 0.4 ml/min, and injection volume = 10 µl. Phenolic acids were separated with a binary gradient elution consisting of aqueous 0.1% formic acid as mobile phase A and 0.1% formic acid in methanol as mobile B.

The method was a 25-minute linear gradient elution programmed as follows: 0-3.81 min, 9-14% B; 3.81-4.85 min, 15-15% B; 4.85-5.89 min, 15-15% B, 5.89-8.32 min, 15-17% B; 8.32-9.71 min, 17-19% B; 9.71-10.40 min, 19-19% B; 10.40-12.48 min, 19-26% B; 12.48-13.17 min, 16-28 % B; 13.17-14.21 min, 28-35% B; 14.21-15.95 min, 35-40% B; 15.95-16.64 min, 40-48% B; 16.64-18.37 min, 48-53% B; 18.37-22.53 min, 53-70% B; 22.53-22.88 min, 9% B; 22.88-25.00 min, 9-9% B. The details of the HPLC program are shown in table format in Appendix D.

Phenolic acid in the samples were identified by comparing their retention times with authentic standards and from previous confirmations using MS spectral data using HPLC-QToF-MS (Drawbridge et al., 2021). The phenolic acids were quantified by extrapolating their peak areas from standard curves plotted using authentic standards including vanillic acid, ferulic acid, *p*-coumaric acid, caffeic acid, and sinapic acids within the concentration range 1.563 mg/ml to 25 µg/ml. Vanillic acid was measured at a wavelength of 280 nm and caffeic acid, *p*-coumaric acid, ferulic acid and sinapic acid were measured at 320 nm.

4.2.10. Antioxidant Assay 2,2-diphenyl-1-picryl-hydrazyl **(**DPPH)

The 2,2-diphenyl-1-picryl-hydrazyl (DPPH) assay was done by the method of Apea-Bah, Head, Scales, Bazylo, & Beta, (2020). A 60 µM DPPH solution was prepared in 100% methanol. A standard curve of trolox (6-hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid (Fluka Chemika)) was prepared consisting of 7 concentrations: 0, 50, 100, 200, 400, 600, and 800 micromole per litre. Five microlitres of trolox or sample was mixed with 195 microlitres of DPPH solution in a 96-well microplate and allowed to incubate in darkness for 30 minutes. The plate was read in a microplate spectrophotometric reader at 515 nm. Dilutions (2x and 5x) of intestinal samples were necessary.

4.2.10. Statistical Analysis

The results were presented as mean ± standard deviation (SD) of triplicate analyses. The data was analyzed by analysis of variance (ANOVA) using SAS version 9.1 (SAS Institute Inc., Cary, NC) and where significant differences existed ($p < 0.05$), Tukey's HSD was used for mean comparison.

4.3. Results and Discussion

4.3.1. Bran layer thickness as measured from microCT images

Appendix B shows the 30 locations of bran thickness measurements taken for each kernel from three CT image slices: transverse, sagittal, and coronal. A total of 6 Roseland kernels and 6 Peru-35 kernels were analyzed. The bran layer was distinctly shown in the images and appeared as a more opaque white colour compared to the endosperm due to its more dense structure, and components which absorbed more of x-rays. The results showed that bran layer thickness of individual kernels of Roseland and Peru-35 were relatively uniform throughout the kernel. For example, the thickness measured at the widest part of the kernel was of similar thickness as the bran located near the narrow tip of the same kernel. Slight variations in bran thickness is likely due to variation and irregularities in the aleurone cell layers (Sapirstein, 2016). The average bran layer thickness of Roseland was 116.52 ± 12.22 μm which was significantly greater than that of Peru-35 (100.67 ± 9.33 μm) (Table 4.1). These bran measurements included the aleurone and pericarp layers.

Table 4.1. Bran layer thickness (µm) measured from microCT images. Bran includes aleurone layer and pericarp layers.

Variety	Bran Layer Thickness (µm)
Roseland	116.52 ± 12.22 [A]
Peru-35	100.67 ± 9.33 [B]

In wheat the thickness of grain tissues are as follows: aleurone (up to 65 µm), seed coat (5-8 µm) and outer pericarp (15-30 µm) (Sapirstein, 2016). Since barley has two to four layers of cells in the aleurone layer, the thickness of the aleurone layer in barley is greater than that in wheat. The aleurone layer thickness in hulled barley was previously reported to range between 100 and 200 µm (Lombi et al., 2011). The aleurone layer of Roseland and Peru-35 could not be clearly distinguished and/or measured in the CT images obtained, thus only the total bran (pericarp plus aleurone) was measured. However, since the aleurone layer spatially makes up most of the bran (Lombi et al., 2011), the aleurone layer in these barley varieties may be around 100 µm as well. An understanding of the bran layer characteristics including chemical composition as well as structure such as thickness is important. The bran layer thickness has implications on kernel hardness and consequently milling, pearling and other processing. Furthermore, when considering the consumption of whole grain barley, the bran layer thickness impacts cooking duration, and contributes significantly to the nutritional value of the grain. No literature could be found on the total bran layer thickness of barley, however, it has been reported that the first pearled fractions up to 18.8% of the barley kernel weight is primarily the bran layer (Madhujith et al., 2006). Therefore,

this work is one of the first to report the bran layer thickness of barley and its measurement in further work involving barley cultivar characterization and bran analysis is justified.

4.3.2. Level of free and bound phenolic acids throughout *in vitro* digestion of cooked barleys

The phenolic acid content was examined at four stages of digestion: 1) half-way through simulated gastric digestion, 2) after simulated gastric digestion, 3) half-way through simulated small intestinal digestion, and 4) after simulated intestinal digestion. Previous work has focused on gastric and intestinal phase endpoints and there is a need for digestion kinetic studies to understand the release of phenolic acids from wheat (Tian et al., 2021) and other whole grains. The free phenolic extracts contained soluble phenolic compounds. The bound extracts, obtained through alkaline hydrolysis followed by ethyl acetate extraction, contained insoluble bound phenolic compounds which could have comprised of esterified, or ether bound phenolic acids. Much of this fraction, however, are likely phenolic acids in the esterified form since it is known that the bran and aleurone layers of barley contain phenolic acid residues esterified to arabinoxylans (Izydorczyk et al., 2014). Tables 4.2 and 4.3 show the free and bound phenolic acid content of the cooked barley varieties (Peru-35 and Roseland respectively) at different stages of digestion. The free phenolic acid content represents the bioaccessible amount that is available for absorption. Therefore, the level of bound phenolic acids at the end of digestion represents the portion that was not bioaccessible and would continue to the large intestine *in vivo*. Although the phenolic compounds in the bound fraction are not bioaccessible in the small intestine, they may provide health benefits in the lower bowel. Further research is needed to understand the extent of the bioactivity of insoluble bound phenolics *in vivo*.

Five phenolic acids were identified in the barley extracts at the four digestion timepoints by comparison to reference standards in the HPLC: vanillic acid, caffeic acid, *p*-coumaric acid, ferulic

acid, and sinapic acid. Peru-35 had significantly greater levels of phenolic acids overall compared to Roseland. Peru-35 and Roseland contained all five of the identified phenolic acids in both free and bound forms, except sinapic acid, which was only found in bound form.

Throughout digestion, bound vanillic and caffeic acids were not detected beyond half-way through the gastric phase. The amounts found are shown in Tables 4.2 and 4.3 but this discussion will focus on ferulic acid, since it was detected and measured in all extracts at all four digestion stages. Ferulic acid was the most abundant phenolic acid in both barley varieties. Throughout digestion, the level of free ferulic acid in Peru-35 samples slightly increased throughout each remaining stage of digestion, but the increase was non-significant. An upward trend throughout the rest of digestion was noted, however. Contrarily, a downward trend throughout digestion was observed for free ferulic acid content in the Roseland digests; however, the decrease was non-significant. Therefore, the level of free ferulic acid remained constant throughout digestion of both barley varieties.

Table 4.2. Phenolic acid content in the cooked hulless barley variety, Peru-35, at different stages of digestion†. Food stability controls (FSC) (contained no digestive enzymes), and pH controls (no pH adjustment), were included for the final gastric and intestinal stages.

		Vanillic acid		**Caffeic acid**		***p*-Coumaric acid**		**Ferulic acid**		**Sinapic acid**	
Digestion Stage		Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound
Half Gastric		6.15 ± 0.78 ^{B}	5.00 ± NA	2.75 ± 0.16 ^{B}	4.23 ± NA	2.31 ± 0.17 ^{B}	6.33 ± 4.50 ^{C}	3.42 ± 0.21 ^{B}	85.92 ± 114.72 ^{B}	-	4.24 ± NA
Gastric		5.85 ± 1.79 ^{B}	5.56 ± 0.59	3.29 ± 0.62 ^{B}	6.52 ± 2.89	2.15 ± 0.32 ^{B}	2.85 ± 1.30 ^{C}	3.79 ± 1.66 ^{B}	102.97 ± 121.89 ^{B}	-	5.18 ± 4.75
	FSC (no enzymes)	49.5 ± 3.62 ^{A}	8.67 ± NA	19.27 ± 6.74 ^{A}	11.21 ± 11.89	17.63 ± 6.49 ^{A}	2.51 ± 0.59 ^{C}	17.62 ± 1.95 ^{A}	179.90 ± 277.3 ^{B}	-	25.86 ± NA
	pH control (no acid)	6.75 ± 0.56^{B}	5.74 ± NA	2.89 ± 0.24 ^{B}	7.47 ± NA	2.39 ± 0.13 ^{B}	1.99 ± 0.06 ^{C}	3.92 ± 0.39 ^{B}	7.86 ± 1.81 ^{B}	-	10.03 ± NA
Half Intestinal		5.74 ± 0.50 ^{B}	-	2.60 ± 0.10 ^{B}	-	2.29 ± 0.42 ^{B}	2.05 ± 0.11 ^{C}	3.96 ± 1.06 ^{B}	9.64 ± 0.84 ^{B}	-	-
Intestinal		5.23 ± 0.21 ^{B}	8.73 ± 2.77	3.02 ± 0.12 ^{B}	35.89 ± 0.23	2.20 ± 0.15 ^{B}	37.09 ± 0.57 ^{A}	4.08 ± 1.31 ^{B}	862.19 ± 4.43 ^{A}	-	6.83 ± 1.07
	FSC (no enzymes)	9.26 ± 1.96 ^{B}		4.47 ± 0.94 ^{B}	2.87 ± NA	4.17 ± 0.83 ^{B}	2.90 ± 0.66 ^{C}	4.93 ± 0.76 ^{B}	39.28 ± 9.64 ^{B}	-	5.20 ± NA
	pH control (no base)	5.95 ± 0.89 ^{B}		3.06 ± 0.40 ^{B}	16.69 ± 5.70	2.49 ± 0.32 ^{B}	17.77 ± 2.32 ^{B}	4.48 ± 0.85 ^{B}	489.26 ± 68.08 ^{A,B}	-	13.37 ± 0.72

† Four timepoints during digestion were examined: 1) "Half gastric", collected at t = 1 hr represents the contents of a samples taken halfway through the gastric digestion phase, 2) "Gastric", collected at t = 2 h represents the samples taken at the end of gastric digestion, 3) "Half intestinal", collected at t = 3 h represents the samples taken halfway through small intestinal digestion, and 4) "Intestinal", represents the samples collected at the end of small intestinal digestion (t = 4 h). †† Values within the same column with the same letter are not significantly different ($P < 0.05$). 'NA' indicates 'not applicable', '-' indicates 'not detected'.

Table 4.3. Phenolic content in the cooked hulless barley variety, Roseland, at different stages of digestion †. Food stability controls (FSC) which contained no digestive enzymes, and pH controls (no pH adjustment), were included for the final gastric and intestinal stages.

		Vanillic acid		**Caffeic acid**		***p*-Coumaric acid**		**Ferulic acid**		**Sinapic acid**	
Digestion Stage		Free	Bound	Free	Bound	Free	Bound	Free	Bound	Free	Bound
Half Gastric		5.54 ± 0.91 ^{C}	-	3.25 ± 1.31 ^{B}	-	4.29 ± 1.10 ^{C}	2.14 ± 0.27 ^{B}	4.60 ± 1.33 ^{B}	4.44 ± 0.03 ^{B}	-	-
Gastric		5.84 ± 0.41 ^{C}	5.32 ± NA	2.61 ± 0.31 ^{B}	-	4.11 ± 0.52 ^{C}	2.50 ± 0.25 ^{B}	4.85 ± 0.90 ^{B}	17.11 ± 2.76 ^{B}	-	-
	FSC (no enzymes)	28.66 ± 1.47 ^{A}	11.24 ± 3.39	13.97 ± 2.46 ^{A}	14.32 ± 6.33	23.75 ± 0.05 ^{A}	17.15 ± 3.32 ^{A}	34.18 ± 8.73 ^{A}	398.84 ± 81.5 ^{A}	-	14.62 ± 6.25
	pH control (no acid)	5.73 ± 0.30 ^{C}	-	-	-	2.98 ±0.28 $^{C, D}$	1.99 ± 0.16 ^{B}	4.43 ± 0.15 ^{B}	5.22 ± 0.90 ^{B}	-	-
Half Intestinal		5.02 ± 0.07 ^{C}	-	2.53 ± NA^{B}	-	2.57 ± 1.17 $^{C, D}$	2.04 ± 0.11 ^{B}	3.85 ± 1.03 ^{B}	8.56 ± 0.94 ^{B}	-	-
Intestinal		4.77 ± 0.29 ^{C}	8.37 ± NA	-	19.43 ± NA	1.63 ± 0.42 ^{D}	8.22 ± 8.88 ^{B}	2.46 ± 0.42 ^{B}	143.3 ± 0.38 ^{A}	-	14.45 ± NA
	FSC (no enzymes)	10.59 ± 0.73 ^{B}	7.25 ± NA	6.12 ± 1.15 ^{B}	12.49 ± NA	8.74 ± 0.25 ^{B}	2.08 ± 0.25 ^{B}	5.49 ± 0.44 ^{B}	9.64 ± 5.12 ^{B}	-	12.78 ± NA
	pH control (no acid)	5.23 ± 0.45 ^{C}	5.15 ± NA	2.51 ± 0.02 ^{B}	5.27 ± NA	2.54 ± 0.09 $^{C, D}$	2.28 ± 0.07 ^{B}	4.33 ± 0.92 ^{B}	10.35 ± 9.86 ^{B}	-	2.96 ± NA

† Four timepoints during digestion were examined: 1) "Half gastric", collected at t = 1 hr represents the contents of a samples taken halfway through the gastric digestion phase, 2) "Gastric", collected at t = 2 h represents the samples taken at the end of gastric digestion, 3) "Half intestinal", collected at t = 3 h represents the samples taken halfway through small intestinal digestion, and 4) "Intestinal", represents the samples collected at the end of small intestinal digestion (t = 4 h). †† Values within the same column with the same letter are not significantly different ($P < 0.05$). 'NA' indicates 'not applicable', '-' indicates 'not detected'.

The level of free ferulic acid in the digesta represents the bioaccessible portion of the total ferulic acid in the sample. In the previous chapter it was determined that ferulic acid bioaccessibility was greater in Peru-35 than Roseland. Taking this into consideration along with the bran layer thickness measurements determined here, preliminary findings suggest there may be an association between bran layer thickness and ferulic acid bioaccessibility. Peru-35 had a thinner bran layer and a higher ferulic acid bioaccessibility, compared to Roseland which had a thicker bran and lower ferulic acid bioaccessibility. Notably a similar trend was observed for *p*-coumaric acid, in that its bioaccessibility was significantly higher in Peru-35, which had a thinner bran layer. The association of bran layer thickness with phenolic acid bioaccessibility requires further investigation.

In terms of bound ferulic acid, the level decreased following the first stages: oral digestion and half-way through gastric digestion in both Peru-35 and Roseland, however, the drop was substantially greater in Roseland despite cooked Roseland containing a similar starting amount of bound ferulic acid as cooked Peru-35 (110.09 ± 5.17 and 141.35 ± 7.33 µg/g d.w.b, respectively). The difference in bound ferulic acid extractability following the first stages of digestion highlight the uniqueness of these varieties in terms of grain matrix and strength of their cell wall structure. Throughout the rest of digestion, from half-way through the gastric phase, to the end of small intestinal digestion (timepoints 1-4) there was a common trend observed in Peru-35 and Roseland: the bound ferulic acid content slightly increased from the half gastric to gastric phase, decreased from the gastric to half intestinal phase, however the changes were not statistically significant. There was a significant increase in the final intestinal phase. This suggests that following full oral, gastric and small intestinal digestion, the barley matrix is loosened and the extractability of bound ferulic acid is increased, resulting in a greater content of bound ferulic acid following digestion

than what was measured in the original cooked barley samples. Notably, similar trends for bound *p*-coumaric acid were found in the digestion of both varieties (Tables 4.2 and 4.3).

Although the level of free ferulic acid did not significantly change throughout digestion of Peru-35 and Roseland, it is possible that during *in vivo* conditions, the loosened bound ferulic acid may be acted upon by other enzymes not present in the current *in vitro* simulation and thereby increase the level of free, bioaccessible ferulic acid. Additionally, the higher level of bound ferulic acid detected after full digestion suggests that a significant portion of ferulic acid continues to the large intestine. Therefore, most of the antioxidant effects from ferulic acid may be elicited in large intestine. In a recent bioaccessibility study of phenolic acids in whole grain wheat, some bound phenolic acids were released into soluble form during upper gastrointestinal (GI) digestion but the authors reported that most of the trans-ferulic acids reached the colon in insoluble-bound form (Tian et al., 2021). While some colonic fermentation experiments on whole grain wheat and barley have shown that colonic microbiota can further release bound phenolic acids including *trans*-ferulic acid (Hole et al., 2012; Tian et al., 2021), a study using human feces for the colonic fermentation reported no increase in soluble phenolic acids; however, this may be due to the rapid metabolism of free phenolic acids to other compounds in the large intestine (Anson et al., 2009). The bioactivity of remaining bound phenolic acids and phenolic acid metabolites in the colon requires further investigation.

4.3.3. Effects of pH and digestive enzymes and fluids on ferulic acid stability

Understanding the role of pH and digestive enzymes can provide information on the stability of the whole grain barley food matrix and the stability of released phenolic acids throughout digestion. To evaluate the stability of barley and ferulic acid in the gastric and intestinal fluids alone, food stability controls (FSC) were included for the gastric and intestinal phases. These

tubes contained the cooked and freeze-dried barley sample, simulated salivary fluid (SSF), and simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), depending on the stage, but they did not contain any enzymes, i.e., alpha-amylase, pepsin, or pancreatin, respectively. The pH was adjusted at the gastric and intestinal stages as per the regular samples. Therefore, the amount of ferulic acid released, if any, would be due to the breakdown of the barley structure by incubation with the simulated digestive fluids. Digestion of these gastric FSC and intestinal FSC tubes were terminated at the end of the gastric and intestinal phases respectively in the same manner as the sample tubes. Following gastric digestion of both Roseland and Peru-35, the lack of enzymes in the FSCs resulted in a significantly higher level of free phenolics for all measurable phenolic acids including ferulic acid. This finding suggests that the action of alpha-amylase and pepsin on starch and protein impacts the barley structure in a way which also leads to the degradation of phenolic acids. The breakdown of starch and protein in the starchy endosperm and aleurone layers may loosen the matrix and allow for the release of bound phenolics or further expose free phenolics. The intact matrix may provide environmental protection of the phenolic acids, thus the lack of enzymes in FSC samples maintained matrix integrity and may explain why phenolic content was higher.

Also, it is possible that the phenolic content was lower in the presence of enzymes due to binding of the phenolic acids with the enzymes, other proteins or starch. Phenolic acids can bind covalently and non-covalently with proteins and starch, and the type of interaction is strongly influenced by the pH of the environment; acidic and neutral conditions favour non-covalent reactions while alkaline conditions favour covalent interactions (Schefer, Oest, & Rohn, 2021). Proteins are the more likely binding partner for phenolic acids in the grain matrix since their structures offer several potential binding sites through hydrophobic and hydrophilic regions, and

nucleophilic side chains of some amino acids (Schefer et al., 2021). Polyphenols can interact with the amylose chains of starch by forming V-type amylose inclusion complexes through hydrophobic interactions, and non-inclusion complexes involving hydrogen bonding between the hydroxyl groups of the phenolic compounds and a hydrophilic component of amylose chains (Amoako & Awika, 2016; Zhu, 2015). Diez-Sánchez, Quiles and Hernando (2021) showed the interactions between blackcurrant pomace polyphenols and macronutrients (starch and protein) and the changes that occurred during simulated *in vitro* digestion. In this study, the starch granules showed fluorescence indicating interactions between the starch polymers and polyphenols (Diez-Sánchez et al., 2021). Phenolic acids, including ferulic acid, caffeic acid, and gallic acid were shown to interact with starch at the α-(1 → 4) glycosidic chains through potential CH-π bonds between starch pyranose rings and phenolic aromatic residues, which may lead to V-type amylose formation (Li, Ndiaye, Corbin, Foegeding, & Ferruzzi, 2020). The authors noted that the phenolic acid-starch complexes formed was dependant on the structures of both the starch and phenolic acids (Li et al., 2020).

Following small intestinal digestion, there were no significant differences between the levels of free ferulic acid in Roseland and Peru-35 digested samples containing enzymes and the respective FSCs, contrary to what was observed at the gastric phase. Thus, an observed difference between free ferulic acid in samples and FSCs at the gastric phase but not at the intestinal phase, suggests that there may be different interactions occurring between free ferulic acid and oral and gastric enzymes, alpha-amylase and pepsin, compared to the interactions with free ferulic acid and pancreatic enzymes.

In terms of bound ferulic acid content among the samples and FSCs there was a similar trend observed at the gastric phase. In Roseland, the lack of enzymes in the FSCs resulted in a

significantly higher level of bound ferulic acid than the gastric samples containing enzymes. However, in Peru-35, although the level of bound ferulic acid in the FSC (179.90 µg/g) was slightly greater than the regular gastric sample tube (102.97 µg/g), the difference was not significant, possibly owing to the large standard deviation among the FSC tubes. This indicates that the level of bound ferulic acid in Peru-35 at the gastric stage is not influenced by alpha-amylase during oral digestion and pepsin during gastric digestion. The differing findings between Peru-35 and Roseland may reflect differences in their grain matrix, specifically the structure of starch and arabinoxylans. In terms of bound ferulic acid content in intestinal digests, the FSCs of the intestinal digests contained significantly less than the enzyme-containing tubes (Tables 4.2 and 4.3). This demonstrates that enzyme action is needed to release the bound ferulic acid from the barley matrix, and it occurs primarily during intestinal digestion rather than gastric digestion. Another possible explanation for the increased level of bound ferulic acid following intestinal digestion is that ferulic acid interacted and bonded to pancreatic enzymes, including pancreatic alpha-amylase, trypsin, and lipase. Previous studies have demonstrated that phenolic acids can bind to pancreatic alpha-amylase, trypsin, and pancreatic lipase (Aleixandre, Gil, Sineiro & Rosell, 2022; Yang et al., 2020; Zheng et al., 2020, Zhou, Zhou, Liu, Zhang, & Cai., 2020). A molecular docking study found that ferulic acid interacts with alpha-amylase and alpha-glucosidase through hydrogen bonding at the active site. Specifically, a single hydrogen bond with alpha-amylase occurred between the C3 and C4 carbonyl group and the amino acid, Gly 334, exhibiting competitive inhibition. Other amino acid residues further away from the active site interacted with ferulic acid through hydrophobic interactions or van der Waals forces, demonstrating non-competitive inhibition. Notably, the competitive interaction formed a stronger, more stable interaction between ferulic acid and alpha-amylase. The inhibitory effects

on alpha-amylase reduced the ability to digest the substrate, starch (Zheng et al., 2020). The same study found that ferulic acid forms three hydrogen bonds and three pi-pi T-shaped interactions with alpha-glucosidase. The authors postulated that ferulic acid binds to a non-catalytic site of the enzyme and its inhibition is through a non-competitive mode (Zheng et al., 2020).

The effect of pH was demonstrated by pH controls which lacked certain pH adjustments. Specifically, the gastric pH control did not contain HCl and the intestinal pH control had undergone pH adjustment with HCl at the gastric phase but did not include sodium bicarbonate during the intestinal phase. Since the digestive enzymes require a certain pH to be active, the results may also reflect the inactivity (or reduced activity) of enzymes. Pepsin requires an acidic pH optimally in the range of 2 to 4. Pepsin becomes unstable above pH 6. Pancreatin acts optimally at a pH of 7, so the intestinal pH control samples which were not adjusted with base (sodium bicarbonate) following acidic gastric digestion, were of a lower pH than optimal for digestion by pancreatin.

Interestingly, in terms of free ferulic acid content, pH controls following oral and gastric digestion as well as following full digestion, were not significantly different than their timepoint counterparts with pH adjustment. However, they were significantly different from their respective FSCs following gastric digestion only. This suggests that there was still pepsin activity in the pH control tubes despite the unadjusted, higher pH during the gastric phase. In terms of the effects of pH on bound ferulic acid content, the absence of acid in the gastric phase resulted in a remarkably lower level of bound ferulic acid in both Roseland and Peru-35 compared to the pH-adjusted samples, however the differences were not statistically significant, likely due to the variation in sample measurements. Similarly, the level of bound ferulic acid following full digestion was lower in the intestinal pH controls (no base added) than the pH-adjusted samples, however the difference

was only significant in Roseland. Previously, it has been shown that different phenolic acids have different stabilities under different pH conditions, and the distribution of -OH atoms of the rings on phenolic molecules may be responsible (Gutiérrez-Grijalva, Angulo-Escalante, León-Félix, & Heredia, 2017). For example, bound ferulic acid is stable at a high pH (Gutiérrez-Grijalva et al., 2017). This helps explain why the level of bound ferulic acid was greater in the pH-adjusted samples which had a higher pH compared to the non-adjusted samples.

4.3.4. Antioxidant activity of the free phenolic extracts

To show that the bioaccessible fraction of the digest indeed has antioxidant properties, the DPPH radical scavenging activity was measured. Table 4.4 shows the radical scavenging capacity of the Peru-35 and Roseland free phenolic extracts obtained at different digestion timepoints, as measured by the DPPH assay. In both Peru-35 and Roseland, the antioxidant capacity significantly decreased from the half gastric to final intestinal stage. Similarly, a study on the effects of digestion on the antioxidant capacity and phenolic content in oregano species found that the DPPH scavenging capacity decreased significantly following *in vitro* digestion (Gutiérrez-Grijalva et al., 2017). Here the free extracts of the fully digested barley samples still showed antioxidant capacity, indicating that these barleys are sources of bioaccessible phenolic compounds which may exhibit some antioxidant capacity *in vivo*.

Table 4.4. DPPH antioxidant assay on phenolic extracts of digesta from Peru-35 and Roseland

	DPPH (micromole TE/g)	
Digestion stage	**Peru-35**	**Roseland**
Half gastric	2.38 ± 0.43 [a]	1.93 ± 0.56 [ab]
Gastric	2.44 ± 0.31 [a]	1.90 ± 0.28 [ab]
Half intestinal	0.60 ± 0.16 [c]	0.45 ± 0.08 [c]
Intestinal	1.53 ± 0.19 [b]	0.33 ± 0.09 [c]

* Values within the same column with the same letter are not significantly different ($P < 0.05$).

4.4. Conclusion

Ferulic acid undergoes structural changes throughout digestion as observed by the level of free and bound ferulic acid at four different timepoints throughout digestion. The release of ferulic acid was strongly influenced by digestive enzymes and minimally affected by pH. The exposure to digestive enzymes reduced the level of free ferulic acid but increased the amount of bound ferulic acid, although the mechanism is unclear. The major release of bound ferulic acid in barley occurred during the final hour of small intestinal digestion. The amount of free, bioaccessible ferulic acid remained constant throughout digestion. The bioaccessibility of ferulic acid may be determined by the bran layer thickness and further investigation is required. This is the first study to investigate the bioaccessibility of ferulic acid in barley at various stages of digestion and the first to examine the bran layer thickness of barley using microCT imaging.

Connections between Chapters 4 and 5

In Chapters 3 and 4, the phenolic acid composition of whole grain barley samples were examined, including studying the effects of cooking and digestion to measure bioaccessibility. The next chapter, Chapter 5, aims to describe potential factors such as grain characteristics which may influence the ferulic acid bioaccessibility. Chapter 5 characterizes pearled fractions of the two varieties selected in Chapter 4, rather than whole grain barley samples. By examining pearled fractions, a more in-depth understanding is gained about the grain matrix of these varieties, and provided a foundation for understanding the role the matrix plays on ferulic acid bioccessibility (measured in Ch. 3) and stability during digestion (examined in Ch. 4). Chapter 5 focuses on the major phenolic acids identified in the previous chapters (ferulic and *p*-coumaric acids), the hydroxycinnamic acid amide discovered in Chapter 3 (*N*1, *N*8- dicaffeoyl spermidine), as well as arabinoxylans. Additionally, Chapter 5 further investigates the bran layer: while Chapter 4 measured the total bran thickness of the selected varieties, Chapter 5 describes the composition of the bran, specifically, the distribution of the major phenolic compounds and arabinoxylans.

CHAPTER 5.

Phenolic Composition and Arabinoxylan Characterization of Pearled Barley Fractions

5. Abstract

Two hulless barley varieties (Peru-35 and Roseland) were pearled into six fractions and characterized in terms of main phenolic acids by HPLC and arabinoxylan content and degree of substitution (arabinose to xylose (A/X) ratio) by gas chromatography. Additionally, the distribution of a new polyamine (*N*8- dicaffeoyl spermidine) identified previously in these barley varieties was reported. An examination of these characteristics is needed in order to determine factors which may influence the bioaccessibility of ferulic and *p*-coumaric acid. Therefore, this work provided information on the arabinoxylan characteristics and phenolic content of the barley varieties, laying the foundation for a future bioaccessibility experiment. The amount of ferulic and *p*-coumaric acids in both varieties was greatest in the outermost fraction and progressively decreased towards the endosperm fraction. The relative abundance of *N*8- dicaffeoyl spermidine was evenly distributed among the intermediary fractions, but greatest in the aleurone-containing fraction (F5) of Peru-35 and in the pericarp-containing fraction (F2) of Roseland. This is the first time the distribution of *N*1, *N*8- dicaffeoyl spermidine in barley is reported. The arabinoxylan content decreased from the outer to inner fractions of the grains and was positively correlated with bound phenolic acid content. The A/X ratio differed among fractions and was strongly correlated with bound *p*-coumaric content. The results from this research provide insight into the composition of pearled hulless barley fractions which can be selected for functional food development based on the desired bioactive components.

5.1. Introduction

There is an increased interest in the consumption of barley due to the health-promoting dietary fibres in barley including beta-glucan and arabinoxylan. Notably, the health claims for the effect of beta-glucan on blood cholesterol lowering (Abumweis, Jew, & Ames, 2010; European Food Safety Authority, 2009; Hughes & Grafenauer, 2021; Wang et al., 2017) prompted the development and valorization of hulless barley varieties, suitable for human consumption. Currently, barley is commercially sold at the market as pearled or pot barley, in which the outer layers have been completely removed or partially abraded off respectively by a pearling machine, to reduce cooking time of the grain and remove the bran and inedible hull. However, removing these outer layers results in a significant loss of minerals, fibre, and antioxidants. Hulless varieties lack a hull but are considered whole grain because the bran is still present. The outer layers of barley are rich in arabinoxylans which are a source of dietary fibre which provides cholesterol lowering, immunomodulatory and prebiotic effects (Mendis & Simsek, 2014), as well as metabolic improvement in diabetes (Garcia et al., 2007). Furthermore, barley contains the second greatest amount of ferulic acid among major cereal crops, following maize (Ndolo & Beta, 2014). The phenolic acids are primarily located in the outer layers of the grain and bound to cell wall carbohydrates such as arabinoxylans and lignin. Therefore, taking together the benefits of fibre and phenolic acids, the arabinoxylans in barley can provide many health benefits (Herrera-Balandrano et al., 2019).

The structure of arabinoxylans in barley varies among grain tissue and cultivars, including differences in the substitution pattern of arabinose units within the xylan chain, the degree of substitution (A/X ratio), and attachment of ferulic or *p*-coumaric acids to the arabinose units (Izydorczyk, 2009; Izydorczyk & Dexter, 2008). The A/X ratio is an indication of the degree

of branching of the arabinoxylan structure which may influence the release of the attached phenolic acids during cooking and digestion. Additionally, our research group previously reported for the first time a polyamine, *N*1, *N*8- dicaffeoyl spermidine, in these barley varieties, which was present in high abundance in the free phenolic extracts, indicating it is bioaccessible and may also provide health benefits. To further characterize this compound in the future, its location and distribution in the grain must be identified for targeted isolation. Therefore, the objectives of this research were to determine the distribution of *N*1, *N*8- dicaffeoyl spermidine in the barley kernel based on industrially relevant pearled fractions in addition to characterizing the ferulic acid, *p*-coumaric acid and arabinoxylan content to describe factors which may influence the bioaccessibility of these phenolic acids in hulless barleys.

5.2. Materials and Methods

5.2.1. Chemicals

Solvents used for extraction of phenolic compounds included HPLC grade methanol, ethyl acetate, and sodium hydroxide from Fisher Scientific (Ottawa ON, Canada). For HPLC analysis, the HPLC grade methanol was used (Fisher-Scientific, Ottawa ON, Canada), and formic acid (Acros Organics, NJ, USA). Phenolic acid standards including vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, and sinapic acid were purchased from Sigma-Aldrich Chemical Company. Sulphuric acid (1M), D-Allose internal standard, sugar standards (xylose and arabinose) were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

5.2.2. Pearling of hulless barley samples

Two hulless barley cultivars, Peru-35 (6-row) and Roseland (2-row) were obtained from the Brandon Research and Development Centre, AAFC, in Brandon, Manitoba, Canada, and harvested in 2012. The samples were pearled with a Satake type TM pearler at the Canadian Grain Commission. The starting weight of each sample was 150 grams and five replicates were done. The pearling products were collected sequentially at 5% weight intervals by successively abrading the kernels up to 25% of their original weight. Therefore, approximately 7.5 grams of product were removed during each pearling increment. The fractions collected were labelled: fraction 1 (F1), representing the first 0-5% product by kernel weight, F2 (5-10%), F3 (10-15%), F4 (15-20%), F5 (20-25%), and lastly, F6 represented the remaining pearled barley (25% pearled). Since 7.5 grams was removed with each pearling increment, the weight of sample remaining after the final pearling (20-25%), was 112.5 grams. The fractions were milled to a fine flour and passed through a 600-micron sieve before being stored in airtight vacuum packing at 4° C until analysis.

5.2.3. Moisture Content

The method used for determining the moisture content of the pearled fractions was that of AACC International Method 44-15.02, Moisture-Air Oven Methods and was done at the Canadian Grain Commission (CGC) in Winnipeg, MB, along with the arabinoxylan determination. By the same method, moisture content in the pearled fractions of Peru-35 was determined in our lab. Two grams of each pearled barley fraction (F1 to F6) were weighed in triplicate into dried aluminum dishes for the analysis. In summary, moisture content was measured as loss in weight of a sample following drying at 130° C in an oven (Heratherm OGS100 Thermo Scientific, Langenselbold, Germany) for exactly 60 minutes, and then cooled

to room temperature in a desiccator (45-60 minutes). The moisture content was expressed as a percentage of the original sample weight.

5.2.4. Determination of total arabinoxylans and A/X ratio

The arabinoxylan content was measured using the methodology outlined by Izydorczyk et al., (2014) and involved hydrolysis of polysaccharides to monosaccharides with 1 M sulphuric acid at 100 °C for 2 hours. Following hydrolysis, the monosaccharides in the samples were reduced and acetylated to alditol acetates according to the method of Englyst & Cummings, (1984). The samples were analyzed by gas chromatography (Agilent 7890 with autosampler) using a Supelco column (SP2380 30m, 0.25mm i.d., 0.2µm film thickness), hydrogen as the carrier gas, run temperature of 225 °C, injector temperature of 270 °C, and flame ionization detector at 270 °C (Izydorczyk et al., 2014). The internal standard was $_{D}$-allose, purchased from Sigma-Aldrich. The total arabinoxylan content was determined as 0.88 × (arabinose content + xylose content) and expressed as a percentage of total dry weight matter.

5.2.5. Phenolic extraction

Free and bound phenolic extraction was performed according to the method of Liu, Qiu, & Beta, (2010) with modification to the duration of hydrolysis with NaOH as reported previously (Drawbridge, Apea-Bah, Silveira Hornung, & Beta, 2021). Briefly, free phenolic compounds were extracted twice using 80% methanol under 1 hour sonication, followed by centrifugation (7000 × g for 10 minutes at 10 °C). The supernatants were collected and combined from each round of extraction, then evaporated to dryness and reconstituted in 50% HPLC grade methanol. The samples were filtered through a 0.22 µm filter into HPLC vials and stored at -20°C until analysis. Bound phenolic compounds were obtained by hydrolyzing the remaining residues from free extraction with 4M NaOH for 14 hours at 4 °C. As conducted in the

previous chapter, the samples were acidified to pH 1.5 with 6M HCl and the phenolic acids extracted three times with ethyl acetate. The upper layer was collected, dried, and reconstituted in 50% HPLC grade methanol. The samples were passed through a 0.22 µm filter prior to analysis and stored at -20 °C.

5.2.6. HPLC analysis

Phenolic acids were identified and quantified following the method outlined by Drawbridge et al., (2021) using a Waters 2695 HPLC system with a photodiode array detector (Waters 2996), and C18 column (Thermo Scientific, Accucore aQ Part no. 17326-103030). Column temperature was set to 35°C and sample temperature was 15°C. The injection volume was 10 µl and the flow rate for the linear gradient elution was 0.4 ml/min consisting of a) aqueous 0.1% formic acid and b) 0.1% formic acid in methanol. The run duration was 25 minutes and the gradient programmed as follows: 0-3.81 min, 9-14% B; 3.81-4.85 min, 14-15% B; 4.85-5.89 min, 15-15% B, 5.89-8.32 min, 15-17% B; 8.32-9.71 min, 17-19% B; 9.71-10.40 min, 19-19% B; 10.40-12.48 min, 19-26% B; 12.48-13.17 min, 26-28 % B; 13.17-14.21 min, 28-35% B; 14.21-15.95 min, 35-40% B; 15.95-16.64 min, 40-48% B; 16.64-18.37 min, 48-53% B; 18.37-22.53 min, 53-70% B; 22.53-22.88 min, 70-9% B; 22.88-25.00 min, 9-9% B. The details of the HPLC program are shown in table format in Appendix D. Ferulic and *p*-coumaric acids in the samples were identified by comparing their retention times with authentic standards and quantified using their peak areas at 320 nm and appropriate standard curves. The polyamine, *N*1, *N*8- dicaffeoyl spermidine, was identified based on the known chromatographic profile of these barley samples (previously determined retention time) and from previous confirmation using MS spectral data using HPLC-QToF-MS (Drawbridge et al., 2021).

5.2.7. Statistics

All analyses were performed in triplicate and statistics were calculated using one-way analysis of variance (ANOVA) on statistical software (SAS Institute Inc., Cary, NC) unless otherwise indicated. Sample means were compared using Tukey HSD method and significant differences were determined at $p < 0.05$. Correlation analysis among parameters were done using Pearson's correlation test. The strength of correlations was classified according to Statology.com, where: an absolute value of the Pearson correlation coefficient (r) < 0.25 = "no relationship", $0.25 < r < 0.5$ = "weak", $0.5 < r < 0.75$ = "moderate", and $r > 0.75$ = "strong". The use of a classification system with four categories as opposed to five or six was chosen in order to more broadly classify the strength of correlations.

5.3. Results and Discussion

5.3.1. Moisture content of pearled barley fractions

The moisture content of the pearled fractions of Peru-35 and Roseland are shown in Table 5.1. These values were obtained from CGC (Winnipeg, MB, Canada) and were used in the determination of total arabinoxylan content, as well as for reporting the phenolic acid content in the barley samples on a dry weight basis. The moisture content for Peru-35 ranged from 10.40% to 11.33%, with the lowest value being in the 25% pearled fraction (F6). Similarly, in Roseland the lowest moisture content was in the F6 fraction and ranged from 10.05% to 11.59 %. These values are consistent with previously reported moisture contents for barley in which the average moisture content of 14 varieties was 12% (Irakli et al., 2020). The values presented here for grain fractions may be slightly lower than those reported for whole grain barley (Irakli et al., 2020) due to more starch in the whole grain, which can hold water.

Table 5.1. Moisture content (%) of the sequentially pearled barley fractions from the varieties Peru-35 and Roseland

Pearled Barley Fraction	Moisture Content, %
Peru-35 0-5%	11.33
Peru-35 5-10%	11.17
Peru-35 10-15%	11.10
Peru-35 15-20%	11.19
Peru-35 20-25%	11.37
Peru-35 25%	10.40
Roseland 0-5%	11.59
Roseland 5-10%	11.59
Roseland 10-15%	11.79
Roseland 15-20%	11.79
Roseland 20-25%	11.97
Roseland 25%	10.05

5.3.2. Arabinoxylan content and degree of substitution

Barley arabinoxylans are mainly located in the cell walls of the outer layers including the aleurone, bran, and husk (Martínez-Subirà et al., 2021), as well as the outer layer of the starchy endosperm (Izydorczyk, 2009). Accordingly, the greatest amount of arabinoxylans in Roseland and Peru-35 were in the outermost layers (F1-F5), with the lowest amounts in the 25% pearled fractions (F6), which contained mostly endosperm. Fractions F1-F5 presumably contained mainly

the pericarp and testa of the grain, with F4 to F5 likely containing aleurone cells. Table 5.2 shows the total amount of arabinoxylans in each of the pearled fractions, as well as the degree of substitution (DS), indicated by the ratio of arabinose to xylose (A/X). Peru-35 had a greater arabinoxylan content than Roseland in each relative fraction (Table 5.2). Total AX content (%) progressively decreased from the outer layer to the inner layers of the grain of both Peru-35 and Roseland. This trend is in accordance with previously reported arabinoxylan contents of pearled barley fractions (Martínez-Subirà et al., 2021). Since the highest amount of arabinoxylans were in the F1 fractions, it can be concluded that the outer pericarp layer of hulless barley is a good source of arabinoxylans.

Table 5.2. Total arabinoxylan content (AX) and A/X ratio of pearled barley fractions obtained after 2 hour and 3 hour hydrolysis

Pearled Barley Fraction	Total AX from 2h hydrolysis (dwb), %	A/X ratio (2h)	Total AX from 3h hydrolysis (dwb), %	A/X ratio (3h)
Peru-35 0-5%	29.08 ± 0.38	0.97	30.88 ± 0.89	0.96
Peru-35 5-10%	22.94 ± 0.39	0.81	24.01 ± 0.61	0.80
Peru-35 10-15%	18.06 ± 0.15	0.73	18.28 ± 0.14	0.73
Peru-35 15-20%	14.19 ± 0.17	0.70	14.91 ± 0.14	0.69
Peru-35 20-25%	11.62 ± 0.16	0.68	11.98 ± 0.01	0.68
Peru-35 25%	5.69 ± 0.08	0.75	5.54 ± 0.28	0.73
		Average = 0.77		Average = 0.77
Roseland 0-5%	20.78 ± 0.05	0.84	22.08 ± 0.08	0.83
Roseland 5-10%	14.83 ± 0.07	0.73	15.36 ± 0.20	0.72
Roseland 10-15%	9.63 ± 0.19	0.69	9.99 ± 0.33	0.68
Roseland 15-20%	6.84 ± 0.03	0.68	6.87 ± 0.40	0.68
Roseland 20-25%	5.50 ± 0.01	0.69	5.66 ± 0.10	0.66
Roseland 25%	2.51 ± 0.09	0.84	2.54 ± 0.00	0.81
		Average = 0.75		Average = 0.73

* AX = arabinoxylan content, A/X = arabinose to xylose ratio (degree of substitution), d.w.b. = dry weight basis

The arabinose to xylose ratio (A/X) is a measure of the degree of substitution and also the amount of branching in the arabinoxylan structure (Malunga, Izydorczyk, & Beta, 2017). The greater the A/X ratio, the higher the degree of substitution and the more branched the structure of the arabinoxylan. The A/X ratio in pearled fractions of Peru-35 was highest in the outermost layer, F1, with a value of 0.97. The A/X ratio decreased in subsequent fractions moving inward towards the endosperm indicating the arabinoxylans had progressively lower degrees of substitution. However, the remaining 25% pearled barley (F6) had a ratio of 0.75. These results are similar to the findings of Izydorczyk & Dexter, (2008) who examined manually separated pericarp, aleurone and endosperm fractions of hulled and hulless barley grains. The authors found that the aleurone layers had the lowest A/X ratios (0.57-0.66), while the pericarp had the highest (1.12-1.15), and the endosperm was in-between (0.81-0.92)(Izydorczyk & Dexter, 2008). In Roseland, after two hours of hydrolysis, the A/X ratio in F1 and F6 were the same (0.84). Similar to Peru-35, the F2-F5 fractions of Roseland had a lower A/X ratio than those of the F1 and F6 fractions. Similar observations have been reported in literature of A/X ratio being greatest in the barley fractions from the outermost grain layers, indicating that the arabinoxylans in the pericarp are more highly substituted (Izydorczyk & Dexter, 2008).

5.3.3. Phenolic content of pearled fractions

Phenolic content of free and bound extracts of pearled barley samples significantly decreased from the outer layer (F1) to the endosperm (F6). The remaining 25% pearled grain (F6) contained very little free and bound ferulic and *p*-coumaric acids. The majority of bound ferulic and *p*-coumaric acid in both Peru-35 and Roseland were in the outermost layer (F1) and decreased progressively through the grain inward. Table 5.3 shows the amount of ferulic and *p*-coumaric acids in the pearled fractions.

Table 5.3. Free and bound ferulic acid and *p*-coumaric acid content of pearled barley fractions

Pearled Barley Fraction	**Ferulic Acid (µg/g)**		***p*-Coumaric Acid (µg/g)**	
	Free	Bound	Free	Bound
Peru-35 0-5%	7.60 ± 0.31 ^{A}	2991.59 ± 133.68 ^{A}	9.65 ± 0.74 ^{A}	164.44 ± 12.87 ^{A}
Peru-35 5-10%	6.04 ± 0.29 ^{B}	2860.92 ± 93.19 ^{A}	9.81 ± 0.28 ^{A}	115.54 ± 3.20 ^{B}
Peru-35 10-15%	4.40 ± 0.86 ^{C}	2349.15 ± 32.47 ^{A,B}	6.77 ± 1.23 ^{B}	84.73 ± 2.55 ^{C}
Peru-35 15-20%	3.76 ± 0.34 ^{C,D}	1885.80 ± 329.52 ^{B,C}	4.98 ± 0.11 ^{B,C}	57.47 ± 13.06 ^{C,D}
Peru-35 20-25%	4.06 ± 0.77 ^{C,D}	1637.79 ± 236.44 ^{C}	4.67 ± 1.13 ^{B,C}	45.79 ± 6.77 ^{D}
Peru-35 25%	2.57 ± 0.12 ^{D}	3.14 ± 0.64 ^{D}	2.71 ± 0.27 ^{C}	-
Roseland 0-5%	2.47 ± 0.71 ^{C,D}	2560.99 ± 150.85 ^{A}	5.38 ± 1.70 ^{A,B}	79.89 ± 5.17 ^{A}
Roseland 5-10%	8.84 ± 0.75 ^{A,B}	2409.08 ± 192.9 ^{A}	6.61 ± 2.11 ^{A,B}	55.77 ± 4.52 ^{B}
Roseland 10-15%	0.80 ± 0.48 ^{D}	1465.25 ± 460.27 ^{A}	3.49 ± 0.01 ^{A,B}	29.06 ± 9.07 ^{C}
Roseland 15-20%	6.96 ± 1.65 B, ^{C}	1064.17 ± 148.02 ^{A}	5.15 ± 1.33 ^{A,B}	21.61 ± 2.72 ^{C}
Roseland 20-25%	12.15 ± 3.26 ^{A}	968.9 ± 26.12 ^{A}	6.67 ± 0.20 ^{A}	19.09 ± 0.46 ^{C}
Roseland 25%	1.57 ± 0.45 ^{D}	3.38 ± 0.88 ^{A}	3.06 ± 0.12 ^{B}	-

* Values within the same column with the same letter are not significantly different ($P < 0.05$).

A similar trend in the distribution of ferulic acid and *p*-coumaric acid in the grain was reported by Gamel & Abdel-Aal, (2012) in a study which examined the whole grain flour and pearled fractions of six different Canadian and Egyptian barley cultivars with various characteristics (hulled, hulless, 2-row, 6-row, waxy and normal). The study reported that ferulic acid was the most abundant phenolic acid in all varieties, representing 75-95% of the total phenolic acids. The hull

fractions contained the greatest amount of ferulic acid. In terms of the Canadian hulless variety examined, the study reported 867.5 µg/g (d.w.b.) in the outermost fraction (pericarp and testa), 511.5 µg/g (d.w.b.) in the middle fraction (parts of aleurone, testa and germ) and only 14.7 µg/g (d.w.b.) in the innermost fraction which represented mainly the white endosperm. These values are lower than the amounts reported in Table 5.3, likely due to cultivar and extraction method differences, however, the downward trend in the level of the major phenolic acids, ferulic and *p*-coumaric acids, from the outer to inner layers of the grain is similar. Additionally, the authors noted that the cultivars grown in Egypt contained higher levels of phenolic acids overall and suggested this may be due to the harsh (hot and dry) growing conditions in Egypt, resulting in increased stress on the plant and production of phenolic acids (Gamel & Abdel-Aal, 2012b). Other studies have reported higher levels of phenolic compounds in outermost layers of pearled barley fractions (Irakli et al., 2020; Madhujith et al., 2006), manually separated botanical barley fractions (Ndolo & Beta, 2014), and pearled wheat fractions (Beta, Nam, Dexter, & Sapirstein, 2005) .

In the free phenolic extracts of both Roseland and Peru-35 there was a prominent peak which corresponded to the hydroxcycinnamic acid amide identified in the whole grains in Chapter 3: *N*1, *N*8- dicaffeoyl spermidine. Since there was no standard available for this compound, the peak areas were reported and the amounts were calculated as ferulic acid equivalents (FAE), shown in Table 5.4. This polyamine was found in all fractions except in F1 of Roseland, and it was more abundant in Peru-35. The amount in Peru-35 ranged from 7.61 to 38.55 microgram per gram FAE. Interestingly, F5 of Peru-35 contained the highest amount of the polyamine, and F1 contained the lowest. The intermediary fractions, F2-F4 were not significantly different ($p < 0.05$), thus the polyamine content was similar throughout these fractions. Since the level of *N*1, *N*8- dicaffeoyl spermidine was more evenly distributed throughout all layers in Peru-35, its relative abundance

compared to ferulic acid therefore increased from the outer to inner layers, since ferulic acid content decreased. This is represented by the peak area percentage for the polyamine of interest. The peak area percent shown in Table 5.4 indicates the percentage the area of the polyamine represents of the entire chromatogram peak area. In Peru-35, there was a significant difference ($p < 0.05$) among all fractions in terms of the peak area percentage for *N*1, *N*8- dicaffeoyl spermidine. A different distribution was found in Roseland. The amount of *N*1, *N*8- dicaffeoyl spermidine in Roseland was highest in F2. Fractions F3-F5 were not significantly different ($p < 0.05$), however the amount in F6, the 25% pearled barley, was significantly lower ($p < 0.05$), and the polyamine was not detected at all in the outer most layer, unlike in Peru-35. Since the *N*1, *N*8- dicaffeoyl spermidine content was greatest in the F5 fraction of Peru-35 and F2 of Roseland, it suggests that this compound may be most abundant in the aleurone of Peru-35 and pericarp of Roseland, although a more precise analysis of the botanical fractions as opposed to pearled fractions is needed. This compound was first identified in whole grain barley in Chapter 3 (Drawbridge et al., 2021), however, this is the first time its distribution in the barley grain is being reported. The differing distribution and abundance of *N*1, *N*8- dicaffeoyl spermidine in various fractions of Roseland and Peru-35 suggests cultivar dependence, although more research is needed on other cultivars to determine whether there are genetic and/or environmental factors.Hydroxycinnamic acid amides are found in many plant species and form through the conjugation of polyamines with a hydroxycinnamic acid, especially ferulic, *p*-coumaric and caffeic acids, and represent the most common type of conjugate polyamines (Lima et al., 2021)(Li et al 2018). The polyamine, spermidine, is known to have anti-oxidant and anti-inflammatory properties, however, more research is needed to determine the extent of these properties in polyamine conjugates such as the one identified here, *N*1, *N*8- dicaffeoyl spermidine. Spermidine exhibits anti-inflammatory

properties in mice by supressing the secretion of proinflammatory cytokines such as tumor necrosis factor- alpha (TNF-α) in the blood. Other hydroxycinnamic acid amides extracted from wolfberries, (*Lycium barbarum*), and the root bark and leaves of wolfberries, exhibited anti-inflammatory properties in an in vitro studies by inhibiting the production of nitric oxide (Wang et al., 2016)(Wang et al 2018). The detailed molecular mechanism of the anti-inflammatory action of hydroxycinnamic acid amides remains unclear and may be investigated using both *in vitro* and *in vivo* models.

Table 5.4. Relative amount of N^1, N^8- dicaffeoyl spermidine in free extracts of pearled fractions (F1-F6) of the barley varieties Peru-35 and Roseland

Pearled Barley Fraction	**N^1, N^8- dicaffeoyl spermidine (µg/g FAE) [d.w.b.]**		**Percentage chromatogram peak area %**	
	Peru-35	**Roseland**	**Peru-35**	**Roseland**
0-5% (F1)	8.58 ± 0.19^{D}	-	9.4 ± 0.8^{F}	-
5-10% (F2)	21.29 ± 0.72^{BC}	7.44 ± 0.40^{A}	16.8 ± 0.6^{E}	6.9 ± 2.8^{B}
10-15% (F3)	25.29 ± 0.30^{B}	4.54 ± 3.19^{AB}	25.3 ± 1.0^{D}	6.1 ± 0.1^{B}
15-20% (F4)	21.77 ± 0.0012^{BC}	6.13 ± 1.31^{A}	34.0 ± 0.8^{C}	5.9 ± 0.04^{B}
20-25% (F5)	43.49 ± 3.64^{A}	6.95 ± 0.22^{A}	43.9 ± 4.1^{B}	5.4 ± 0.1^{B}
25% pearled (F6)	17.47 ± 3.16^{C}	0.64 ± 0.26^{B}	65.3 ± 2.9^{A}	19.1 ± 4.6^{A}

* Values within the same column with the same letter are not significantly different ($P < 0.05$). d.w.b = dry weight basis. FAE = ferulic acid equivalents. '-' indicates 'not detected'

5.3.4. Correlation analysis

Pearson correlation between the major phenolic acids (ferulic and *p*-coumaric acids) and fraction, AX content, and degree of substitution (A/X ratio) are presented in Table 5.5. It was hypothesized that the arabinoxylan content would positively correlate with ferulic and *p*-coumaric acid content. In general, a common trend was found between the pearled fractions (F1 to F6) and the phenolic acid and AX content, with the exception of free ferulic acid and free *p*-coumaric acid in the Roseland variety only. This observation showed a linear decrease in both AX content and bound ferulic and *p*-coumaric acids from the outer pearled fraction (F1) to the 25% pearled barley comprising of the inner portion of the grain (F6). In Roseland, the free ferulic acid content was greatest in the F5 fraction, which may suggest that free ferulic acid within the outer layers is concentrated in the aleurone of Roseland. The second highest level of free ferulic acid in Roseland was in the F2 fraction. Contrarily, there was no trend found between pearled fraction and A/X ratio, however, the A/X ratio decreased slightly from F1 to F5 in both Peru-35 and Roseland. The A/X ratio in F6 of Roseland was comparable to the F1 fraction. The A/X ratio in F6 of Peru-35 was intermediary. The F6 fraction represents the largest portion of the barley grain (75% by weight) and may show a more average A/X ratio compared to the fractions containing just 5% of the grain by weight.

Table 5.5. Correlations between ferulic acid, *p*-coumaric acid, arabinoxylan (AX) content, and degree of substitution (A/X) ratio among pearled barley fractions.

Comparison	**Pearson *r* value**	**R squared value**	**p value**	**Correlation strength**	**Significance at α =0.05**
Correlation between AX content and bound ferulic acid content in Peru-35	.93	0.86	0.007	strong	significant
Correlation between AX content and bound ferulic acid content in Roseland	.95	0.91	0.003	strong	significant
Correlation between AX content and bound *p*-coumaric acid content in Peru-35	.98	0.97	0.002	strong	significant
Correlation between AX content and bound *p*-coumaric acid content in Roseland	.99	0.99	0.0004	strong	significant
Correlation between A/X ratio and bound ferulic acid content in Peru-35	.52	0.27	0.294	moderate	not significant
Correlation between A/X ratio and bound ferulic acid content in Roseland	.009	0.00009	0.99	none	not significant
Correlation between A/X ratio and bound *p*-coumaric acid content in Peru-35	.99	0.98	0.001	strong	significant
Correlation between A/X ratio and bound *p*-coumaric acid content in Roseland	.95	0.9	0.014	strong	significant
Correlation between AX content and free ferulic acid content in Peru-35	.97	0.95	0.00099	strong	significant

Correlation between AX content and free ferulic acid content in Roseland	.13	0.017	0.81	none	not significant
Correlation between AX content and free *p*-coumaric acid in Peru-35	.96	0.93	0.002	strong	significant
Correlation between AX content and free *p*-coumaric acid in Roseland	.37	0.14	0.47	weak	not significant
Correlation between A/X ratio and free ferulic acid in Peru-35	.85	0.72	0.03	strong	significant
Correlation between A/X ratio and free ferulic acid in Roseland	.53	0.28	0.28	moderate	not significant
Correlation between A/X ratio and free *p*-coumaric acid in Peru-35	.72	0.52	0.1	moderate	not significant
Correlation between A/X ratio and free *p*-coumaric acid in Roseland	.33	0.11	0.52	weak	not significant

In terms of phenolic content, the A/X ratio was strongly positively correlated with bound *p*-coumaric content only. There was no correlation between A/X ratio and ferulic acid content. This suggests that the majority of bound *p*-coumaric acid is attached to arabinoxylans, whereas bound ferulic acid may be attached to other carbohydrates in the grain in addition to arabinoxylans. The A/X ratio indicates the degree of substitution of arabinose with xylose units and also indicates the amount of branching in the structure, which may impact the bioaccessibility of any phenolic residues which may be attached. Previous studies have reported a higher degree of branching in the water extractable arabinoxylans in pearled pericarp fractions of barley compared to the

endosperm (Izydorczyk, Jacobs, & Dexter, 2003). *In vitro* digestion studies are needed to determine if there is a correlation between A/X ratio and bioaccessibility of ferulic and *p*-coumaric acid.

Arabinoxylan content was positively correlated with ferulic and *p*-coumaric acids, except for free ferulic acid content in Roseland. The correlation may be partially explained by the fact that ferulic and *p*-coumaric acid in barley are predominantly present in bound form to arabinose units of arabinoxylans via an ester linkage (Izydorczyk, 2009). Additionally, both the free and bound phenolic acids progressively decreased from F1 to F6 overall, as did the total arabinoxylan content. The level of free ferulic acid in Roseland may have been too low to establish a clear correlation.

Correlations were observed between the amount of *N*1, *N*8- dicaffeoyl spermidine and arabinoxylan content in the pearled fractions for both Peru-35 and Roseland. The outcomes of the correlation analysis involving *N*1, *N*8- dicaffeoyl spermidine are shown in Table 5.6. Furthermore, *N*1, *N*8- dicaffeoyl spermidine content was strongly correlated with the A/X ratio (r = -0.76 (Peru-35) and r = 0.81 (Roseland)). These findings suggest that *N*1, *N*8- dicaffeoyl spermidine may be associated with the fibre component of grains, and further research is needed to further localize this compound in the grain and identify any interactions it may have with the polysaccharides. *N*1, *N*8- dicaffeoyl spermidine also strongly correlated with the free ferulic acid content (r = 0.78) and bound ferulic acid content (r = 0.78) in Roseland. Contrarily, in Peru-35, *N*1, *N*8- dicaffeoyl spermidine was only weakly correlated with free ferulic acid content *(*r = -0.43) and showed no correlation with bound ferulic acid content (r = -0.18). Regarding its relationship with *p*-coumaric acid, the *N*1, *N*8- dicaffeoyl spermidine content showed different correlations (weak or strong) depending on the variety and free or bound extract. For instance, there was a strong correlation observed between *N*1, *N*8- dicaffeoyl spermidine and free *p*-coumaric acid in Roseland (r = 0.89)

but only a weak (and opposite) correlation in Peru-35 (r = -0.89). Contrarily, there was a weak correlation observed between *N*1, *N*8- dicaffeoyl spermidine and bound *p*-coumaric acid in Roseland (r = 0.40) and a strong correlation in Peru-35 (r = -0.82).

Table 5.6. Correlations among *N*1, *N*8- dicaffeoyl spermidine and AX and phenolic content and A/X ratio in Peru-35 and Roseland

Comparison	**Pearson *r* value**	**R squared value**	**Correlation**
Correlation between *N*1, *N*8- dicaffeoyl spermidine (Peru-35) and AX content	-0.46971	0.220624	weak
Correlation between *N*1, *N*8- dicaffeoyl spermidine (roseland) and AX content	0.659359	0.434755	moderate
Correlation between *N*1, *N*8- dicaffeoyl spermidine (Peru-35) and A/X ratio	-0.76334	0.582683	strong
Correlation between *N*1, *N*8- dicaffeoyl spermidine (Roseland) and A/X ratio	-0.81108	0.657843	strong
Correlation between *N*1, *N*8- dicaffeoyl spermidine (Peru-35) and free ferulic acid	-0.43412	0.188458	weak
Correlation between *N*1, *N*8- dicaffeoyl spermidine (Roseland) and free ferulic acid	0.784904	0.616075	strong
Correlation between *N*1, *N*8- dicaffeoyl spermidine (Peru-35) and bound ferulic acid	-0.17765	0.031559	none
Correlation between *N*1, *N*8- dicaffeoyl spermidine (Roseland) and bound ferulic acid	0.783568	0.613979	strong
Correlation between *N*1, *N*8- dicaffeoyl spermidine (Peru-35) and free *p*-coumaric acid	-0.39362	0.154936	weak
Correlation between *N*1, *N*8- dicaffeoyl spermidine (Roseland) and free *p*-coumaric acid	0.889189	0.790657	strong
Correlation between *N*1, *N*8- dicaffeoyl spermidine (Peru-35) and bound *p*-coumaric acid	-0.82314	0.677567	strong
Correlation between *N*1, *N*8- dicaffeoyl spermidine (Roseland) and bound *p*-coumaric acid	0.39912	0.159297	weak

The production of both polyamines, such as *N*1, *N*8- dicaffeoyl spermidine, and phenolic compounds including ferulic and *p*-coumaric acids, are increased in response to pathogenic attacks. *N*1, *N*8- dicaffeoyl spermidine was only found in the free phenolic extracts, so its correlations with arabinoxylan content, bound ferulic acid in Roseland and bound *p*-coumaric acid in Peru-35 is interesting and suggests there may be some influence of *N*1, *N*8- dicaffeoyl spermidine on the arabinoxylan structure or phenolic content. Previous research in plants has shown that polyamines conjugate with phenolic compounds in response to stress as protective mechanism, for example, spermidine has been found conjugated with hydroxycinnamic acids (Legaz, De Armas, Piñón, & Vicente, 1998). Therefore, where correlations were observed between *N*1, *N*8- dicaffeoyl spermidine and bound ferulic and *p*-coumaric acid, it is possible that some of the bound phenolic acids were conjugated to *N*1, *N*8- dicaffeoyl spermidine. Since the results differed between Peru-35 and Roseland, further research examining the relationship between *N*1, *N*8- dicaffeoyl spermidine, arabinoxylan and phenolic acids in additional barley varieties is justified, as well as a comparison of barley grown under different environmental conditions.

5.3.5. Discussion on the potential of select grain fractions for functional foods

This research contributes to the advancement of hulless barley varieties and the furtherance of barley in grain-based functional foods. Pearling is a common method of processing barley and characterizing pearled fractions in terms of health promoting constituents is important in identifying and selecting fractions for functional foods or nutraceuticals. There is a correlation between ferulic and *p*-coumaric acids in pearled barley fractions and antioxidant activity (Gamel & Abdel-Aal, 2012b), confirming the contribution of these phenolic acids to the health benefits of consuming barley and warranting further research on food-grade barley varieties. Pearling eliminates the hull, but as consumers interest in barley increases the demand of barley products,

there is potential to increase the use of conventional roller milling techniques on hulless varieties (Zheng, Li, & Wang, 2011). In addition to the arabinoxylan and major phenolic compounds quantified here, beta-glucan is another major health-promoting compound in barley. Barley beta-glucan has been associated with blood cholesterol-lowering (Abumweis et al., 2010; Wang et al., 2017). The distribution of beta-glucan in barley is opposite of that of arabinoxylan and phenolic acids, with the greatest amount found in the endosperm, and progressively decreases towards the outermost layers. Therefore, fraction selection for fibre-rich foods will be based on the desired level of arabinoxylan or beta-glucan.

In terms of the polyamine, *N*1, *N*8- dicaffeoyl spermidine, the relatively even distribution among intermediary pearled fractions means that these fractions will provide similar amounts of this compound, which could be an added benefit to choosing these fractions, as they also contain a good balance of arabinoxylans and beta-glucan as well. For *N*1, *N*8- dicaffeoyl spermidine-enriched fractions, F5 of Peru-35 and F2 of Roseland contained the greatest amount and could potentially be targeted for functional food development. However, more research is needed on the health benefits of *N*1, *N*8- dicaffeoyl spermidine before selecting pearled fractions based on its content. As mentioned earlier, the next step in this research area is to determine the bioaccessibility of phenolic acids in the pearled fractions and determine if there is a correlation between A/X ratio and release of ferulic and *p*-coumaric acids, which would facilitate selection/screening of pearled fractions with more bioaccessible phenolic acids based on A/X ratio, and aid in an understanding of the role of the grain matrix in bioaccessibility.

5.4. Conclusion

The amount of ferulic acid and *p*-coumaric acid in two hulless barley varieties decreased from the outermost pearled fraction containing mainly pericarp and progressively decreased towards the endosperm fraction. The arabinoxylan content also progressively decreased from the outer to inner fractions of the grains and was positively correlated with the bound phenolic acid content. Additionally, this research characterized pearled fractions of the barley varieties in terms of A/X ratio, which was strongly correlated with bound *p*-coumaric content. The A/X ratio demonstrated further differences among fractions and is the foundation for future bioaccessibility studies on these pearled hulless barley varieties. Additionally, the abundance of a polyamine, *N*1, *N*8-dicaffeoyl spermidine, was reported as ferulic acid equivalents and was found evenly distributed among the intermediary fractions. However, its content was greatest in F5 of Peru-35 and F2 of Roseland. This is the first time the distribution of *N*1, *N*8- dicaffeoyl spermidine in barley is being reported. The results from this research provide insight into the composition of pearled hulless barley fractions which can be selected for functional food development based on the desired bioactive components.

CHAPTER 6

Summary and Conclusions

6. Research Summary and General Conclusions

The aim of this dissertation was to determine the bioaccessibility of ferulic acid in hulless barley and examine grain matrix characteristics which may influence its bioaccessibility. The main topics of the research included 1) profiling hulless barley varieties in terms of major phenolic acid content, 2) the effect of boiling on the level of phenolic acids, 3) the effect of digestion and the bioaccessibility of ferulic acid, 4) grain-matrix characteristics including bran thickness, bran composition, and arabinoxylan structure which may influence the release of ferulic acid during digestion. Upon analyzing the free phenolic extracts, a polyamine, found in relatively high abundance compared to ferulic acid, was identified for the first time in whole grain barley. This compound, identified as N_1, N_8, dicaffeoyl spermidine, was quantified in the whole grain barley as well as pearled fractions, since it may also provide antioxidant and anti-inflammatory effects. The significance of identifying this bioactive compound may include the promotion of these barley varieties for human consumption and new barley products.

The results reported in Chapter 3 provide new insights into the bioaccessibility of the major phenolic acids found in four hulless barley varieties. Hulless barley is food-grade, and its development and production increased in recent years due to the promotion of barley consumption for its beta-glucan content and the health claim approved by Health Canada regarding blood cholesterol lowering. Therefore, it was important to further examine these hulless varieties in terms of their ferulic acid content. It is well known that whole grain cereals contain ferulic acid which contributes to the health benefits associated with whole grains, however, much of the ferulic acid is located in the hull, which is absent in these varieties. Furthermore, bioaccessibility studies on carotenoids and phenolic acids in wheat emerged; however, there were no bioaccessibility studies on ferulic acid in hulless barley, making this the

first published work on the subject. The results showed that the barley varieties, despite not having a hull, still contained a high level of free and bound phenolic compounds. Ferulic and *p*-coumaric acids were the most abundant phenolic acids, and their bioaccessibilities differed among varieties, with one variety, Peru-35, having significantly greater ferulic acid bioaccessibility.

The results reported in Chapter 4 provide information on the bioaccessibility of ferulic acid throughout digestion to determine when the majority of this acid is released during the digestive process. The chapter also investigates factors which may affect the release of ferulic acid including digestive enzymes and pH. For this study, two (Peru-35 and Roseland) of the four varieties evaluated in Chapter 3 were selected. Peru-35 had the greatest ferulic acid bioaccessibility while Roseland contained high levels of ferulic and *p*-coumaric acids, the two most abundant phenolic acids in barley, and the second highest ferulic acid bioaccessibility of the four varieties. This research showed that the release of ferulic acid was strongly influenced by digestive enzymes and minimally affected by pH. The release of bound ferulic acid in barley occurred mainly during the final hour of small intestinal digestion. The level of bioaccessible ferulic acid decreased during the first hour and then remained constant throughout digestion. The antioxidant capacity of the free phenolic extracts significantly decreased from the half gastric to final intestinal stage, however, the fully digested barley samples exhibited antioxidant capacity, indicating that these barleys are sources of bioaccessible phenolic compounds which may elicit some antioxidant properties *in vivo*. Additionally, the bran layer thickness of these barley varieties was measured by a novel application of microCT imaging. The bioaccessibility of ferulic acid may be influenced by the bran layer thickness, therefore further studies on bran microstructure are needed.

Chapter 5 examined the ferulic and *p*-coumaric acid content and arabinoxylans in pearled fractions of the two barley varieties studied in Chapter 4 (Peru-35 and Roseland). This chapter focused on *p*-coumaric acid in addition to ferulic acid because it is also known to be bound to the arabinoxylans in barley. Since the phenolic acids are not bioaccessible in this bound form, the arabinoxylans were characterized in terms of total content and degree of substitution (A/X ratio). It is my hypothesis that the amount and structure of arabinoxylans to which ferulic acid is bound to impacts its release from the grain matrix and ultimately its bioaccessibility. Therefore, this work provided information on the arabinoxylan characteristics and phenolic content of the barley varieties, laying the foundation for a future bioaccessibility experiment. Pearled fractions were examined rather than the whole grain flour because it allows for observing any distinct differences in the arabinoxylan A/X ratio, whereas measuring the A/X ratio in the whole grain produces only an average result based on all the types of arabinoxylans in the grain. The results from this study showed that the A/X ratio differed among fractions and was correlated with bound *p*-coumaric content. Arabinoxylan content decreased from the outer to inner pearled fractions of the grains and a similar trend was observed for bound phenolic acid content. Ferulic and *p*-coumaric acid content progressively decreased from the fraction obtained from the outermost layer of the grain to the innermost pearled fraction. *N*1, *N*8- dicaffeoyl spermidine was evenly distributed among the intermediary fractions, but greatest in the aleurone-containing fraction of Peru-35 and in the pericarp-containing fraction of Roseland. This is the first time the distribution of *N*1, *N*8- dicaffeoyl spermidine in whole grain barley has been examined.

In summary, Chapter 3 provided insight into the bioaccessibility of phenolic acids in four hulless barley varieties. Chapters 4 and 5 delved deeper into understanding the bioaccessibility of the major phenolic, by determining the ferulic acid content throughout digestion, and examining

factors which may potentially impact bioaccessibility. These factors included physical grain traits such as bran layer thickness and arabinoxylan characteristics, as well as the role of enzymes and pH in the release of ferulic acid from the grain matrix. Taking the findings of the three experimental chapters together, it can be concluded that hulless barley is a source of bioaccessible ferulic acid. The level of free and bound ferulic acid varies throughout digestion due to the stability in the digestive environments and the many interactions ferulic acid has with components of the grain matrix.

6.2. Summary of Contributions

The main contributions of the work presented in this thesis are as follows:

- Determined the bioaccessibility of the major phenolic acid (ferulic acid) in food-grade, hulless, barley varieties, further promoting the consumption of whole grain barley
- Provided an understanding of the ferulic acid content in barley throughout simulated digestion
- Demonstrated the novel application of microCT imaging to non-destructively measure bran layer thickness of whole grain barley kernels
- Discovered a polyamine, *N*1, *N*8- dicaffeoyl spermidine, in whole grain barley, including reporting its distribution within the grain as observed in pearled barley fractions
- Provided insight into the composition of pearled hulless barley fractions which can be selected for functional food development based on the desired bioactive components.

These contributions have led to the publication of one journal article in Food Chemistry (358 (2021) https://doi.org/10.1016/j.foodchem.2021.129905) and 5 conference abstracts.

6.3. Suggestions for Future Research

The findings of this research showed that ferulic acid bioaccessibility in hulless barley differs among varieties. This research examined the effects of digestion on ferulic acid and began the investigation into factors which may influence ferulic acid bioaccessibility. Therefore, further research is needed to determine the extent to which specific grain matrix components and characteristics influence ferulic acid bioaccessibility. Is the bioaccessibility primarily determined by bran layer thickness, for example? Is the bioaccessibility of ferulic acid primarily determined by structural characteristics of the arabinoxylans present in the grain? To answer these questions, a future digestion experiment on the pearled barley fractions, and a greater range barley type (increased sample size) for correlation analysis is needed. Specifically, further research is needed to:

1) Determine if there is a correlation between ferulic acid bioaccessibility and bran layer thickness, total arabinoxylan content, and A/X ratio. The preliminary findings in this dissertation point towards the ferulic acid bioaccessibility being greater in grains with a thinner bran layer. Additionally, the preliminary findings suggest that a thicker bran layer does not necessarily indicate a greater arabinoxylan and phenolic content.

2) Further understand the stability of ferulic acid during digestion and the role the grain matrix plays in its stability, including studying the interactions ferulic acid has with barley proteins and starch in the digestive environment.

3) Determine the bioavailability of ferulic acid from hulless barley using animal models.

4) Investigate the synergy, if any, between ferulic acid and *N*1, *N*8- dicaffeoyl spermidine.
5) Isolate *N*1, *N*8- dicaffeoyl spermidine from the phenolic extracts of barley to determine its antioxidant and anti-inflammatory properties and the health benefits it may provide.

As with all studies, this research is not without limitations. The main limitation of this work is that a static digestion model was used to simulate human gastrointestinal digestion and determine the ferulic acid bioaccessibility. While other models such as semi-dynamic and dynamic exist, they are costly and difficult to conduct. However, all simulated *in vitro* models have been shown to have good correlation with *in vivo* results. Future studies could employ an *in vivo* method to confirm the results and include a bioavailability study to better understand the contribution of barley ferulic acid to human health. The digestion method followed the protocol outlined by INFOGEST, which was formulated based on the international consensus among experts. The method provides suggestions for different types of foods (liquid beverage, solid bolus, high-fat foods etc.) and research objectives (e.g., bioaccessibility, digestibility studies, etc.). The method employed in this research used select recommendations from the publication pertinent to the experiment. However, since digestive conditions in this study were found to significantly influence the ferulic acid content during digestion, it is recommended that future studies aim to standardize the digestion method. A method could be formulated specifically for the simulated digestion of cereal grains to facilitate comparisons among studies.

Future digestion studies should include the oral phase, as was done in this research project because of the impact of adding the oral phase to digestion experiments, especially for investigation of starch-rich foods such as cereal grains. Including the oral phase replicates the start of starch hydrolysis in the mouth by alpha-amylase. The oral phase also simulates mastication to

reduce particle size. Previously, inclusion of the oral phase was considered optional (Minekus et al., 2014), however, the current Infogest method advises that the inclusion of the oral phase is mandatory, regardless of bolus type, i.e. liquid or solid, highlighting the importance of including the oral phase in digestion experiments.

With regards to *N*1, *N*8- dicaffeoyl spermidine, future studies could isolate and purify this compound from barley to create a standard curve in HPLC for the accurate quantification of *N*1, *N*8- dicaffeoyl spermidine, eliminating the need to report the quantity as ferulic acid equivalents. The antioxidant and anti-inflammatory properties of *N*1, *N*8- dicaffeoyl spermidine should be examined, along with any additional spermidine-conjugates or hydroxycinnamic acid amides identified in additional barley varieties.

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APPENDICES

APPENDIX A: Barley Varieties

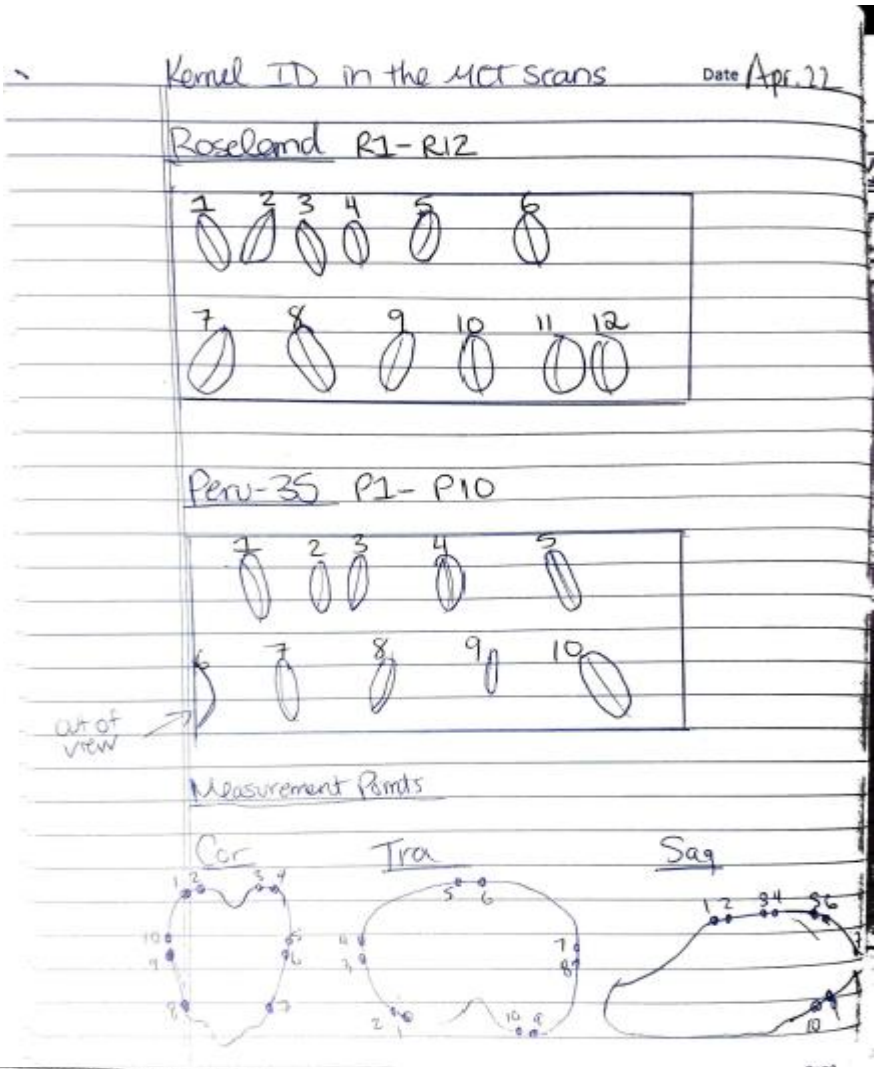
Peru-35

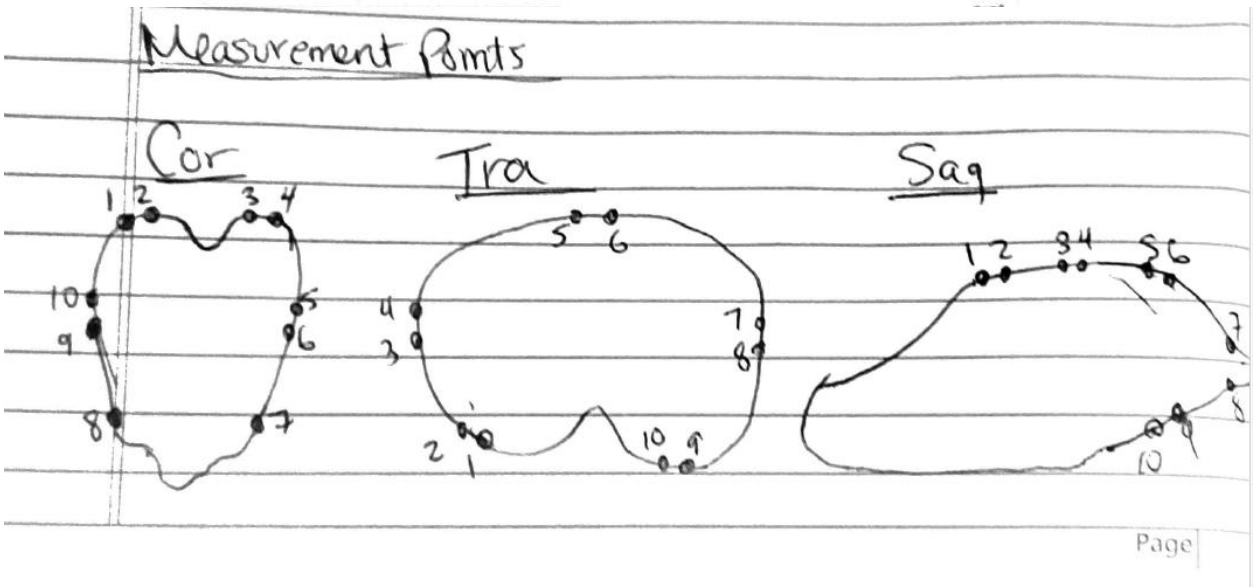
CI1248

Roseland

Atahualpa

APPENDIX B: Locations of Bran Thickness Measurements

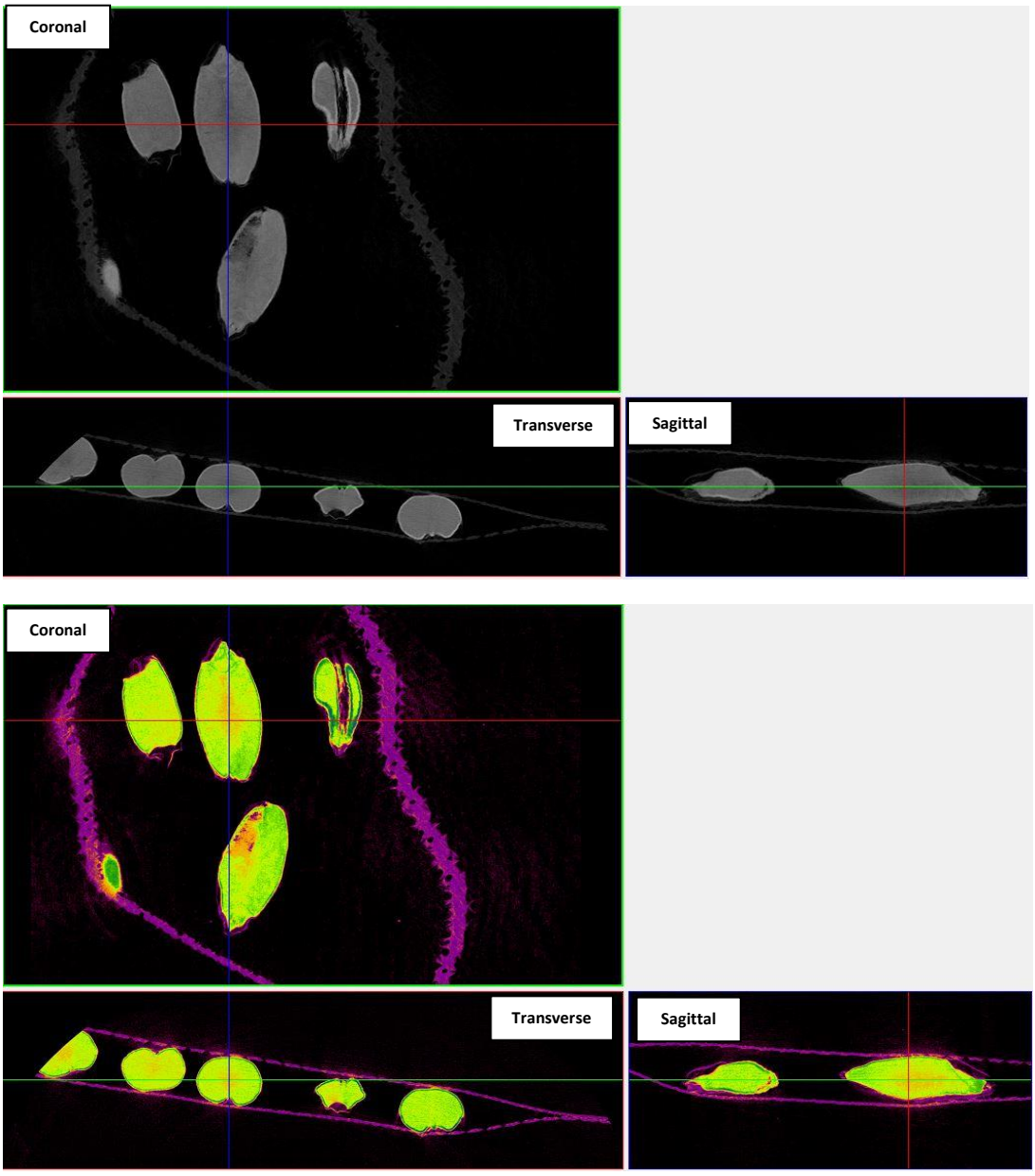




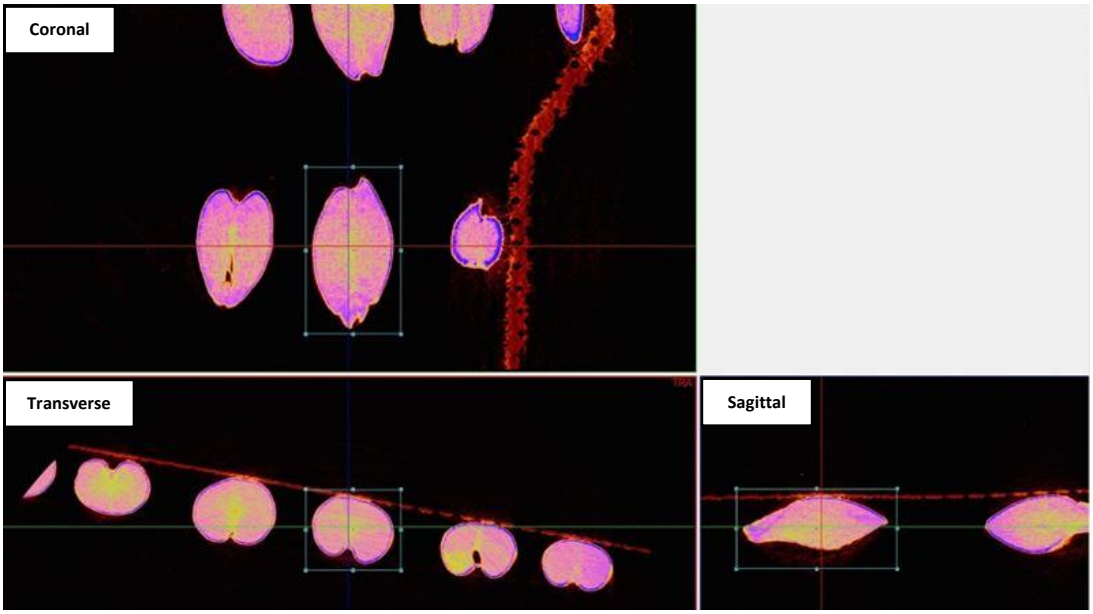
APPENDIX C: Sample microCT Images and Data Set Details

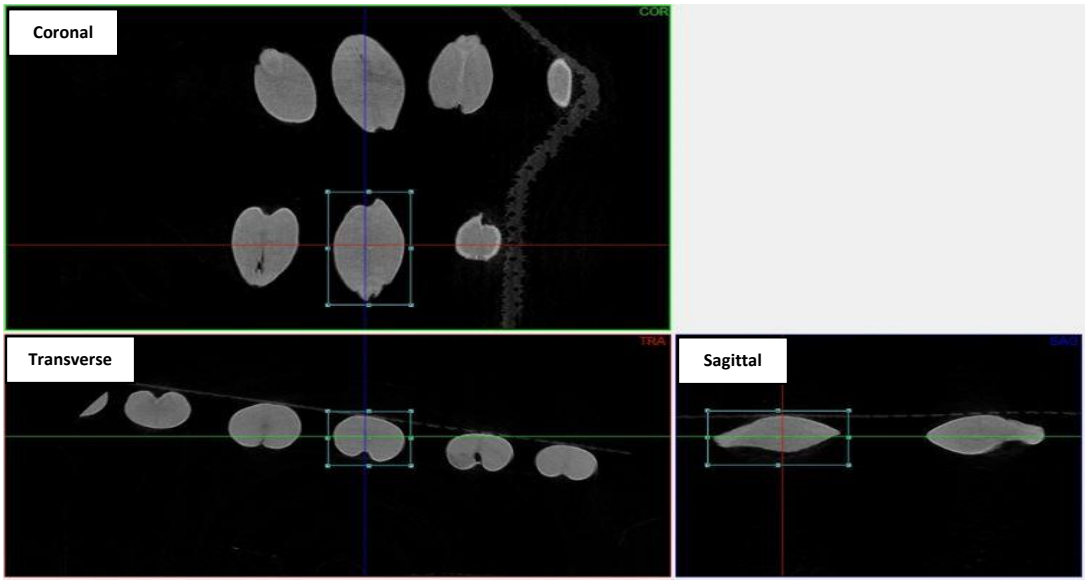
MicroCT image of whole grain Peru-35 kernels shown using CTvox software.

Representative cross-sectional microCT 3D image showing the sagittal, coronal, and transverse slices of a Peru-35 grain kernel, shown in grey scale and colour using DataViewer software.



Representative cross-sectional microCT 3D image showing the sagittal, coronal, and transverse slices of a Roseland grain kernel, shown in grey scale and colour.





MicroCT Imaging Dataset

Dataset	
Description	Value
Image Dimension	3500 x 1056
Pixel Size (original)	8.894, 8.894 micron
Acquisition Information	
Description	Value
Time/Date	May 27, 2021 12:33:21
Scan duration	00:38:37
Scanner	SkyScan1176
Instrument SN	11C0834
Camera type	Princeton Instruments
Source type	PANalytical's Microfocus Tube
Camera pixel	12.640 µm
Camera X/Y ratio	0.999
Camera-source distance	165.226 mm
Object-source distance	118.163 mm
Energy filter	No filter
X-ray source voltage	44.0 kV
X-ray source current	568.0 µA
Optical axis	1338
Exposure time	775.0 ms
Frame averaging	2
Random movement	Unknown
Rotation range	180 degrees
Rotation step	0.300 degrees
Rotation direction	CCW
Reconstruction Information	
Description	Value
Reconstructed by	NRecon(Version: 2.0.0.5)
Time/Date	27 May 2021 12h:39m:10s
Data format	8-bit BMP (NRecon)
Value inversion	Not inverted
Dynamic range	0.001066…0.049738
Post alignment	-2.5
Smoothing width	1
Beam-hardening correction	30
Ring correction	7
Big object	On
Image rotation	0.0 degrees
ROI option	ON
HU conversion	HU = 12.506 * Value + (-930.000)
Defect pixel mask level	50

APPENDIX D: HPLC Program

Table A1. Programmed flow for the HPLC gradient elution used for the separation and analysis of phenolic acids and *N*1, *N*8- dicaffeoyl spermidine in phenolic extracts. The composition of the mobile phase consisted of 0.1% formic acid in water (Mobile phase A) and 0.1% formic acid in methanol (Mobile phase B) over a 25-minute run* with a Waters 2695 HPLC system.

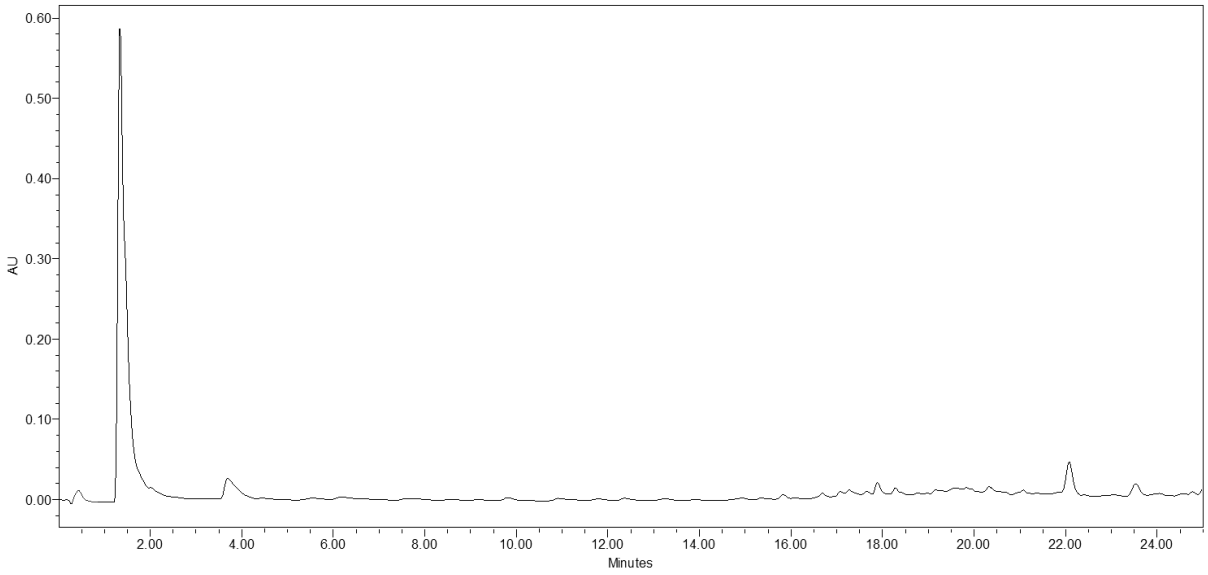
Time (min)	Flow (ml/min)	% A: Water with 0.1% formic acid	% B: Methanol with 0.1% formic acid	Curve
0	0.40	91.0	9.0	6
3.81	0.40	86.0	14.0	6
4.85	0.40	85.0	15.0	6
5.89	0.40	85.0	15.0	6
8.32	0.40	83.0	17.0	6
9.71	0.40	81.0	19.0	6
10.40	0.40	81.0	19.0	6
12.48	0.40	74.0	26.0	6
13.17	0.40	72.0	28.0	6
14.21	0.40	65.0	35.0	6
15.95	0.40	60.0	40.0	6
16.64	0.40	52.0	48.0	6
18.37	0.40	47.0	53.0	6
22.53	0.40	30.0	70.0	6
22.88	0.40	91.0	9.0	6
25.00	0.40	91.0	9.0	11

* **Run Conditions**; HPLC column: C18 (Thermo Scientific, Accucore aQ Par no. 173-103030); Column temperature = 35 °C; Sample temperature = 15 °C; Detector: photodiode array detector (Waters 2996) detected at wavelengths of 280 nm and 320 nm.

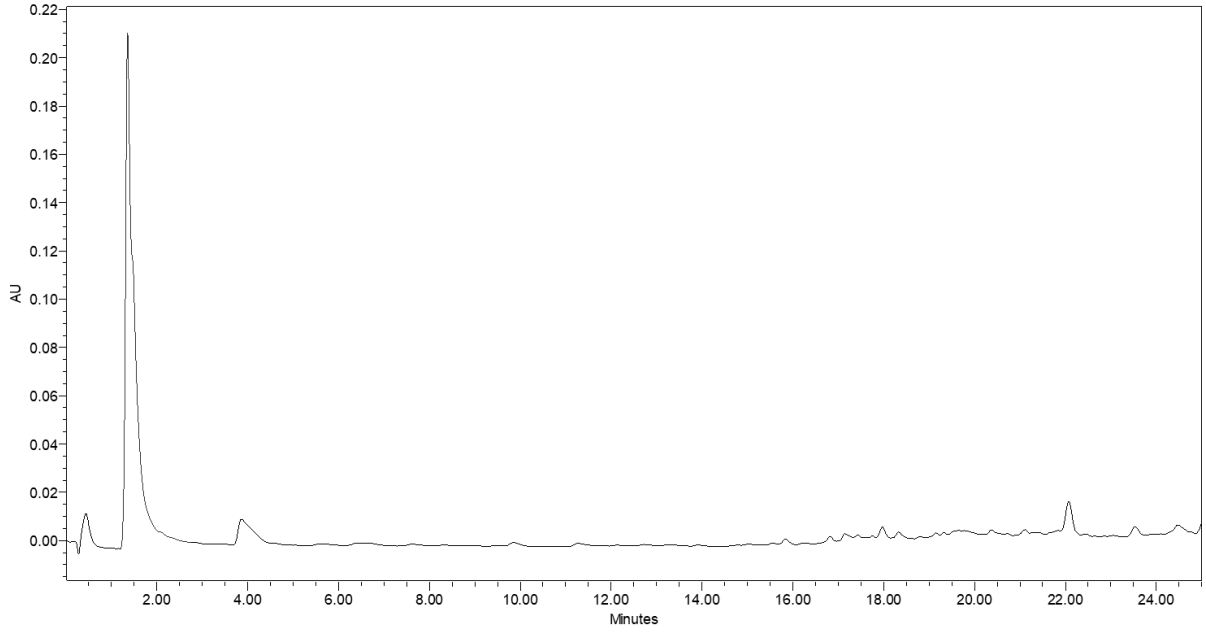
APPENDIX E: Representative chromatograms from Chapter 4

Select chromatograms representative of digestion timepoints presented in Ch. 4: Bioaccessibility of Ferulic Acid in Hulless Barley Varieties at Stages of Simulated *in vitro* Digestion.

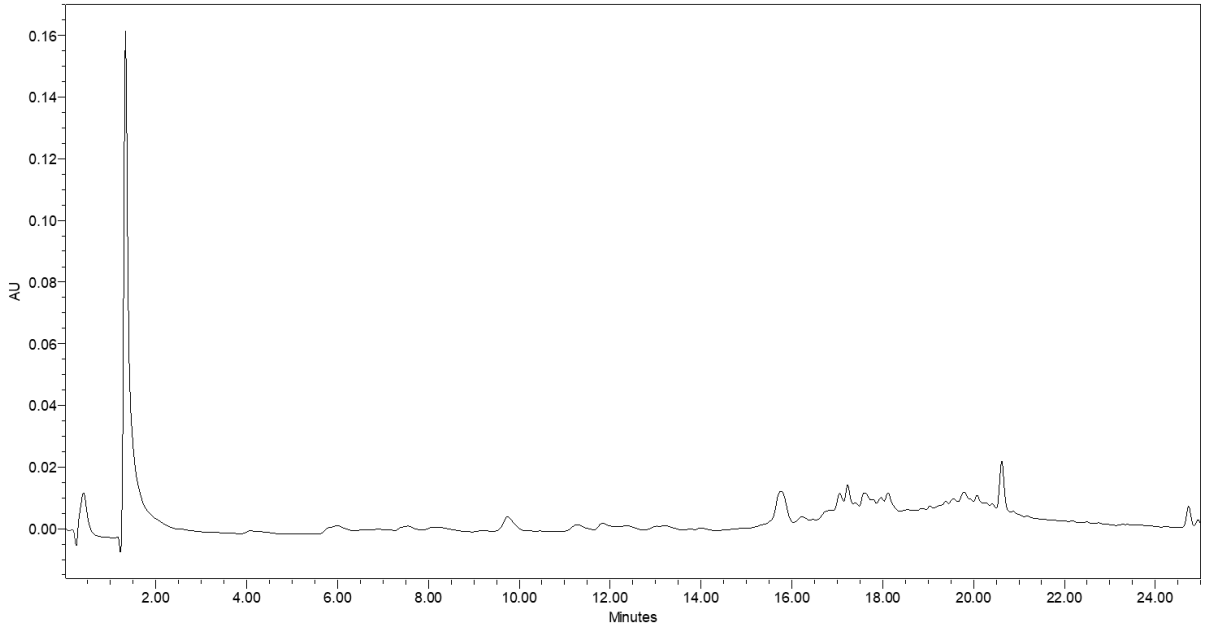
P1halfGastric (Batch 1 Free) 320nm



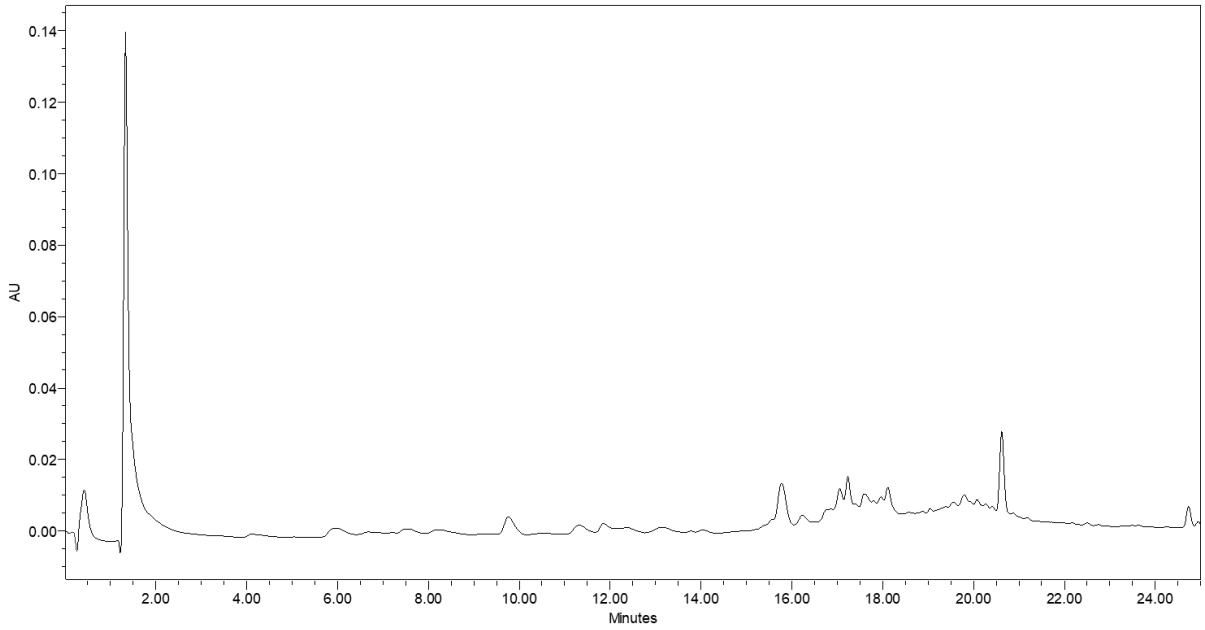
P2Gastric (Batch 2 Free) 320nm



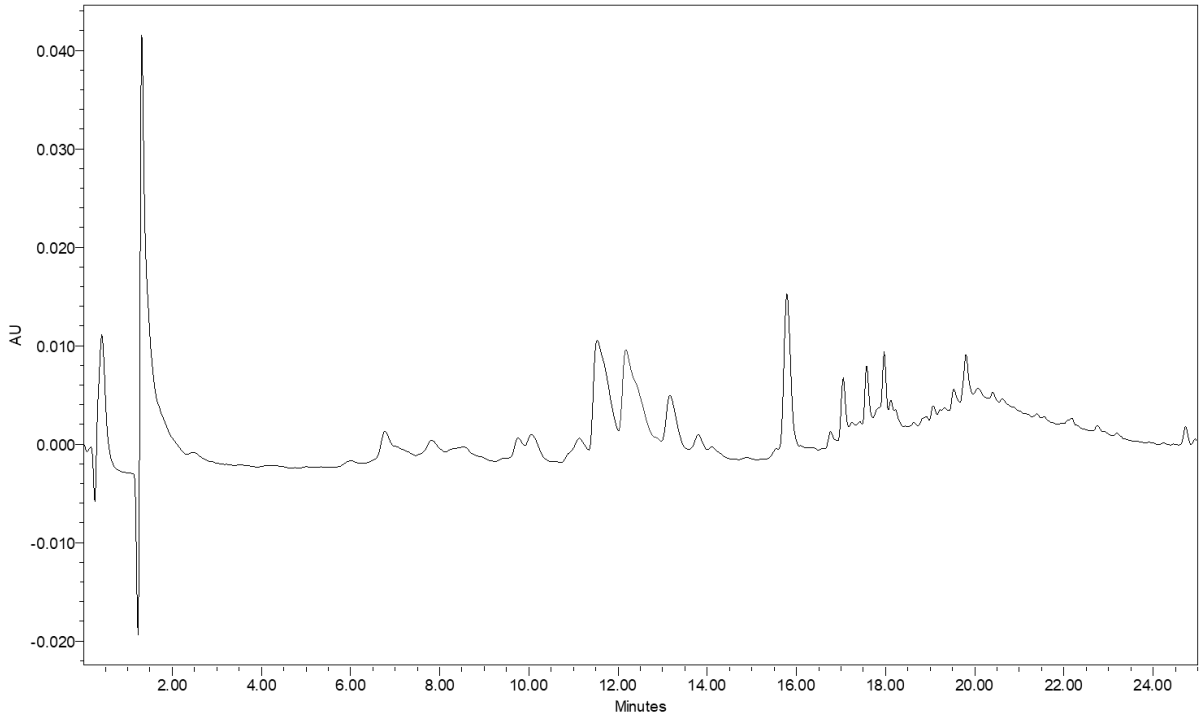
P3HalfIntestinal (Batch 1 Free) 320 nm



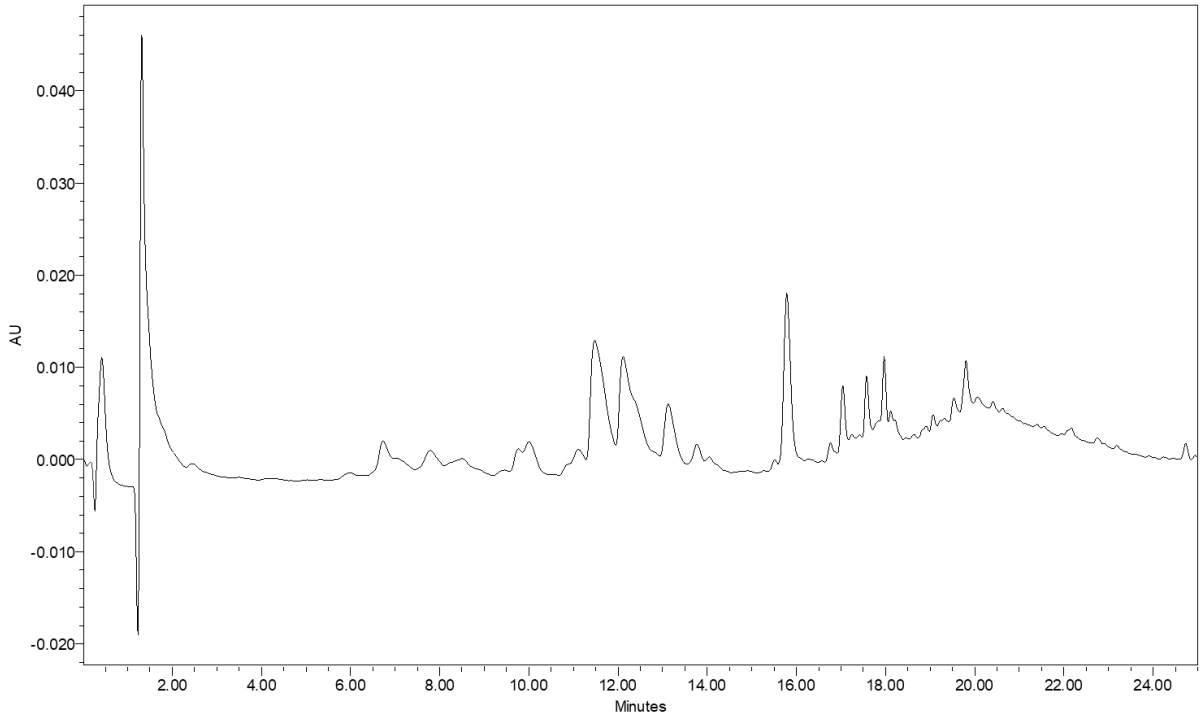
P2Intestinal (Batch 1 Free) 320nm



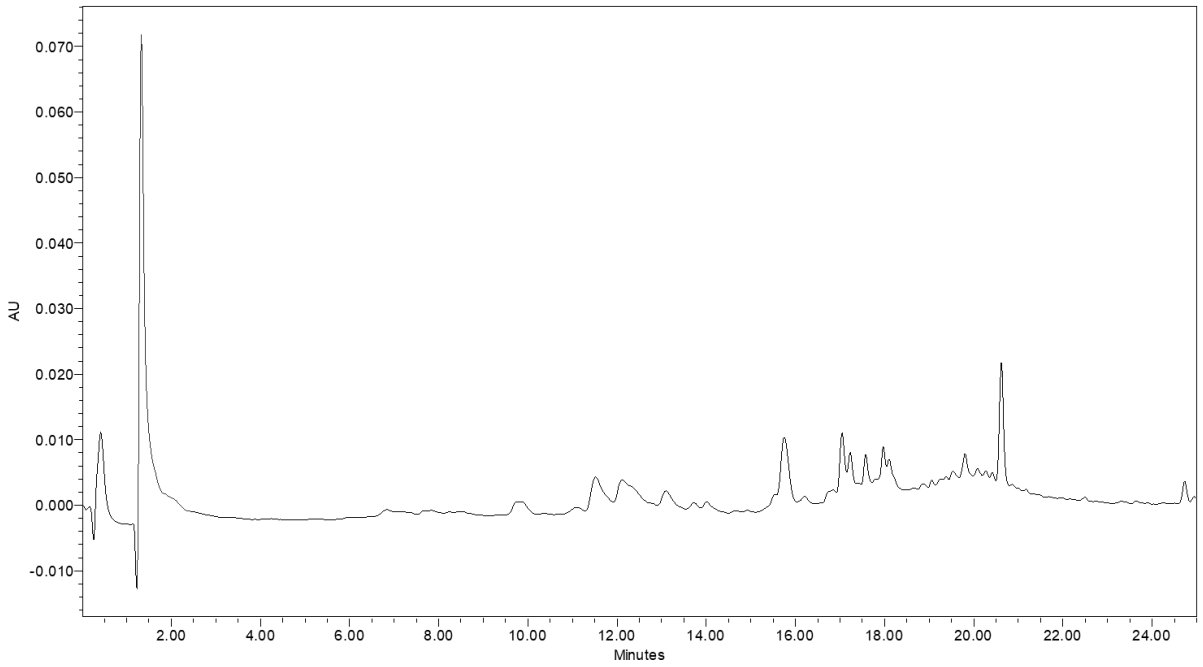
R2halfgastric (Free Batch 2) 320nm



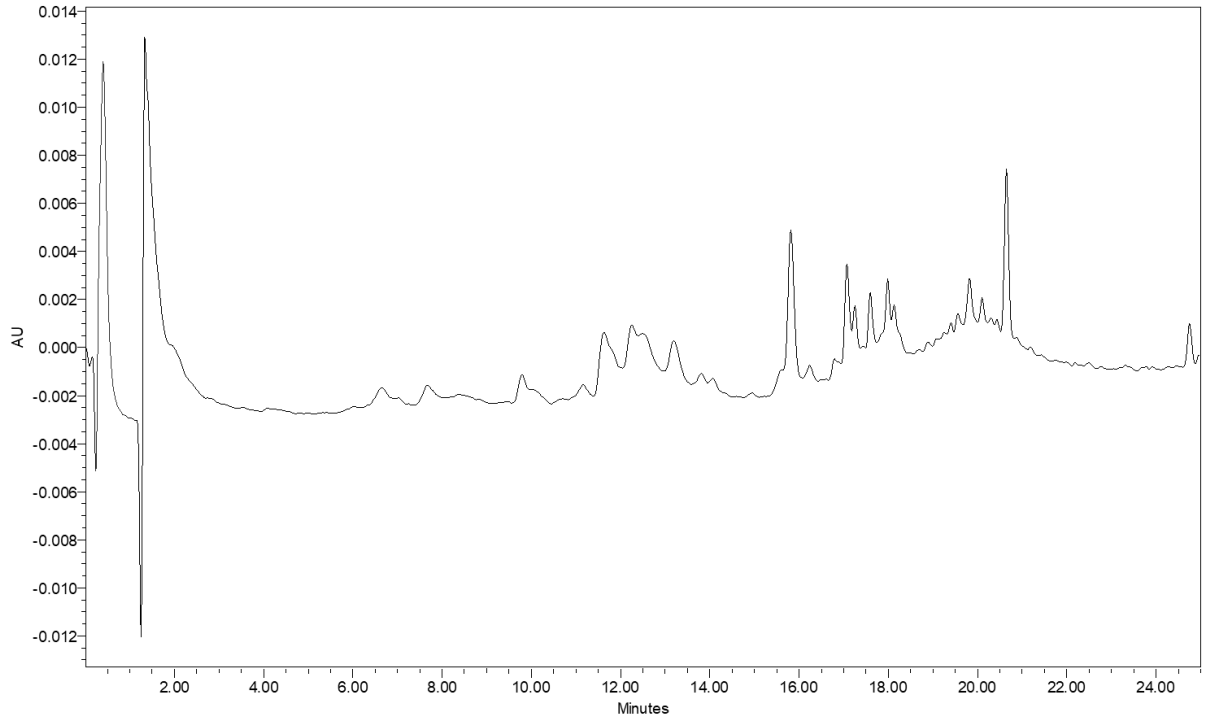
R2Gastric (Free Batch 2) 320nm



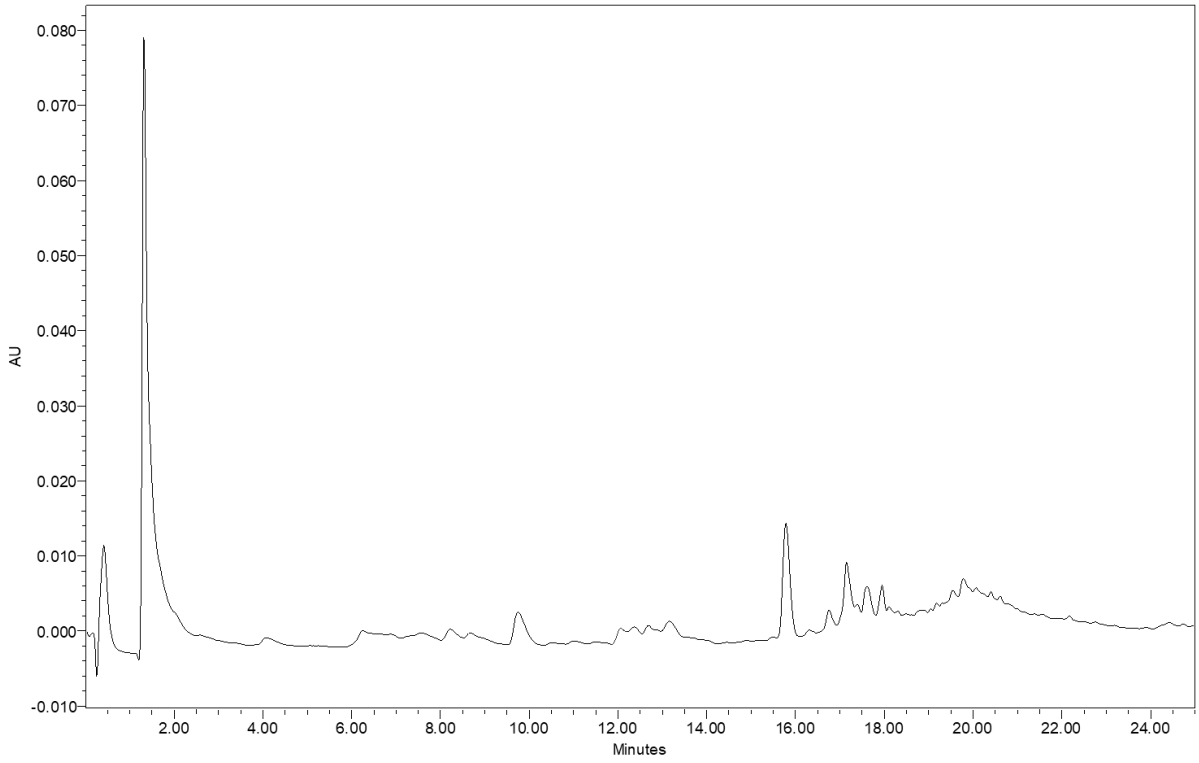
R2HalfIntestinal (Free) 320 nm



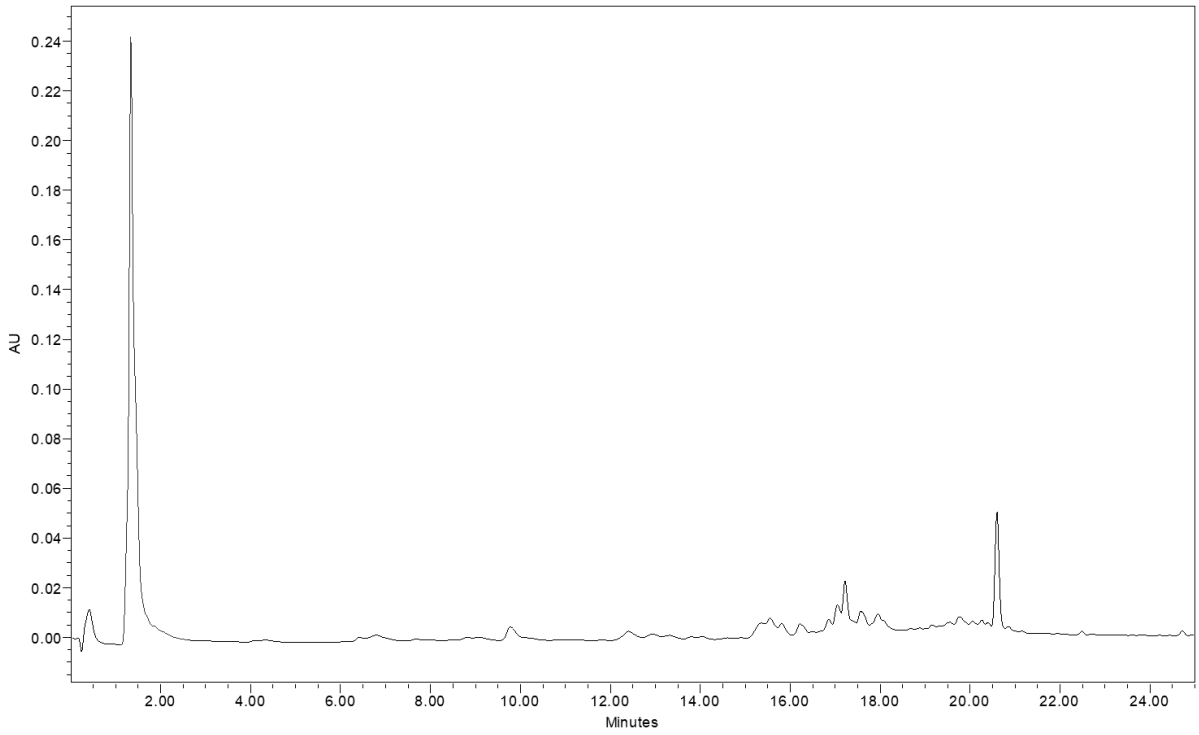
R2intestinal (Free) 320nm



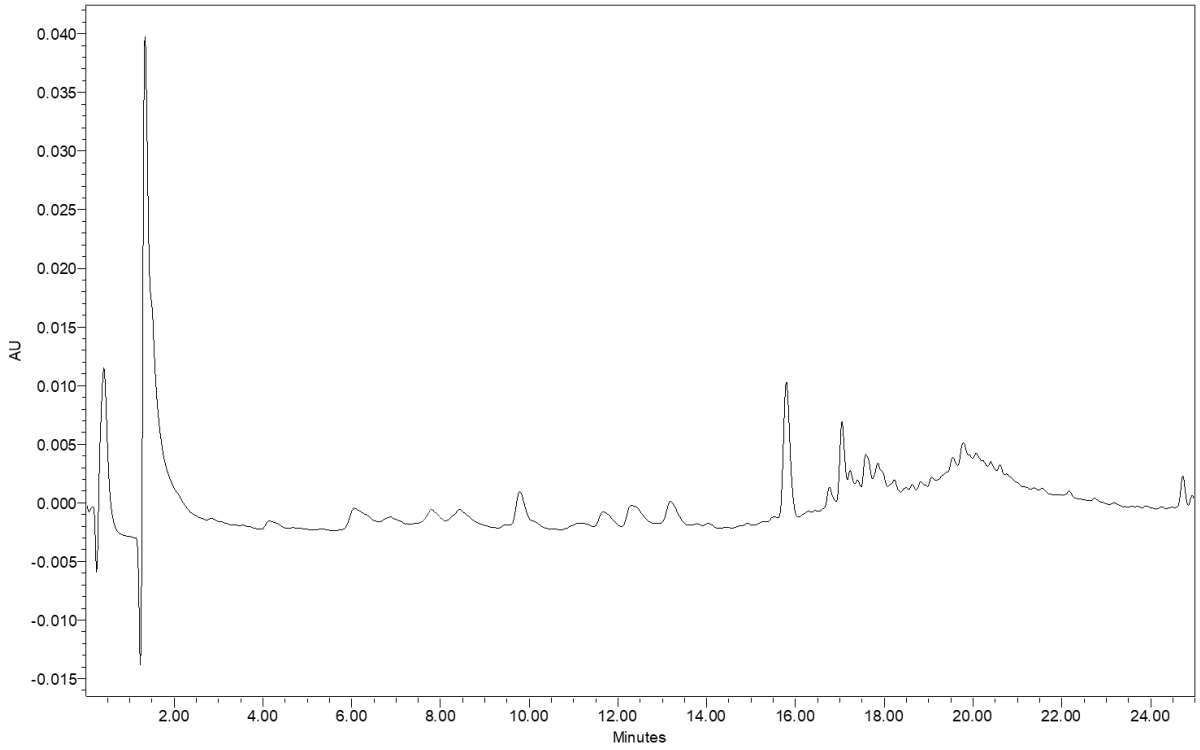
P-FSC-G3 FREE 320 nm



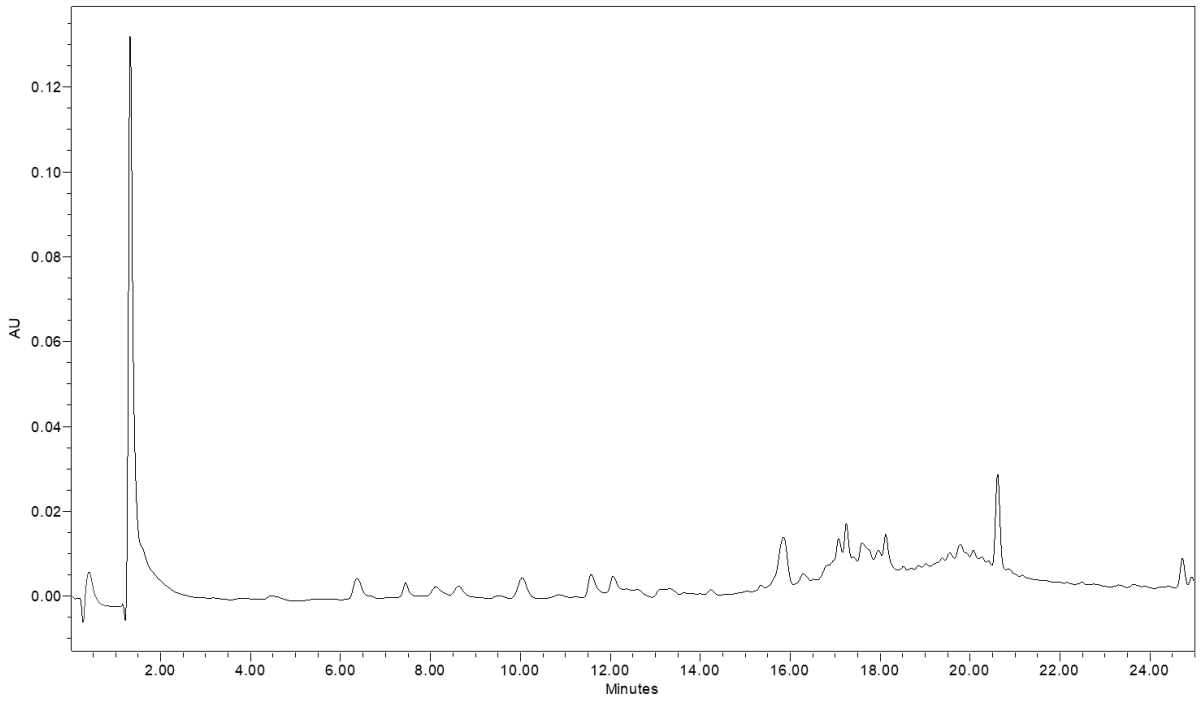
P-FSC-I1 FREE 320nm



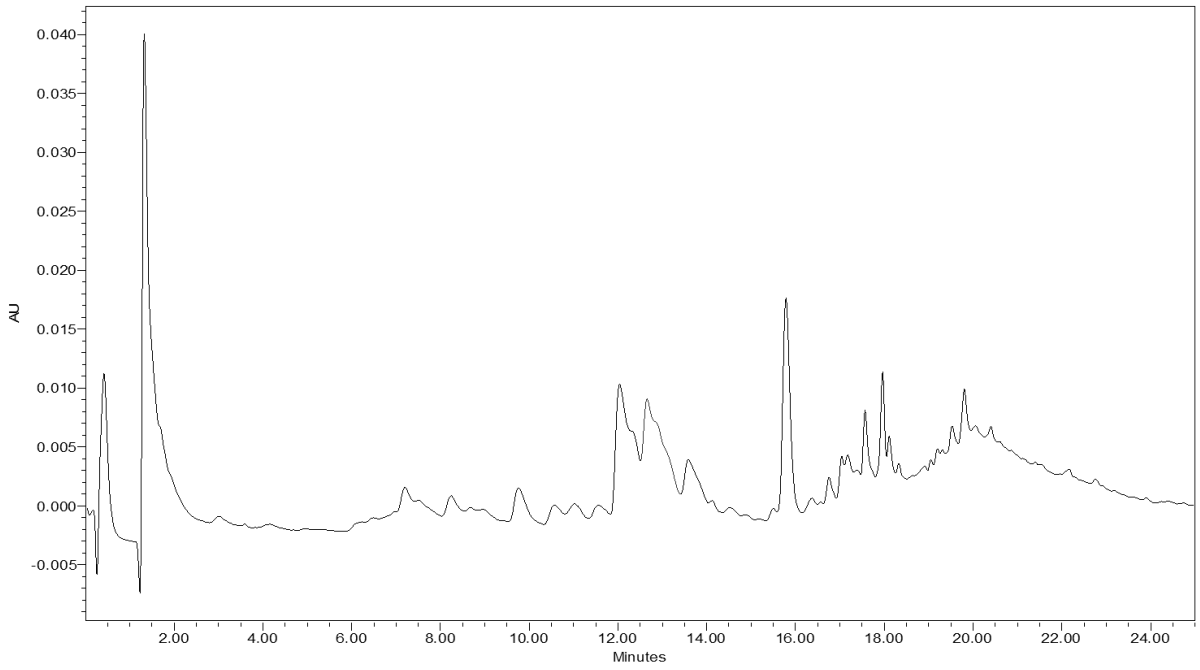
P-pH-G2 Free 320nm



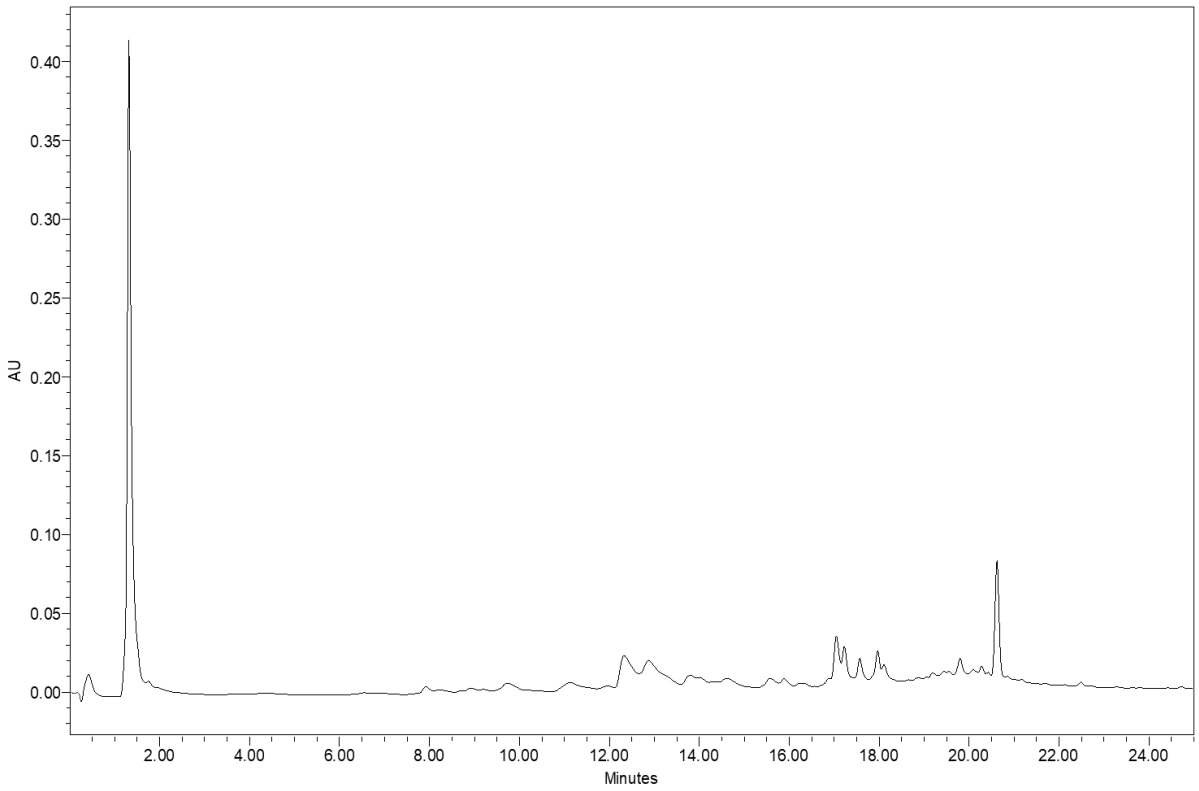
P-PH-I1 FREE 320nm



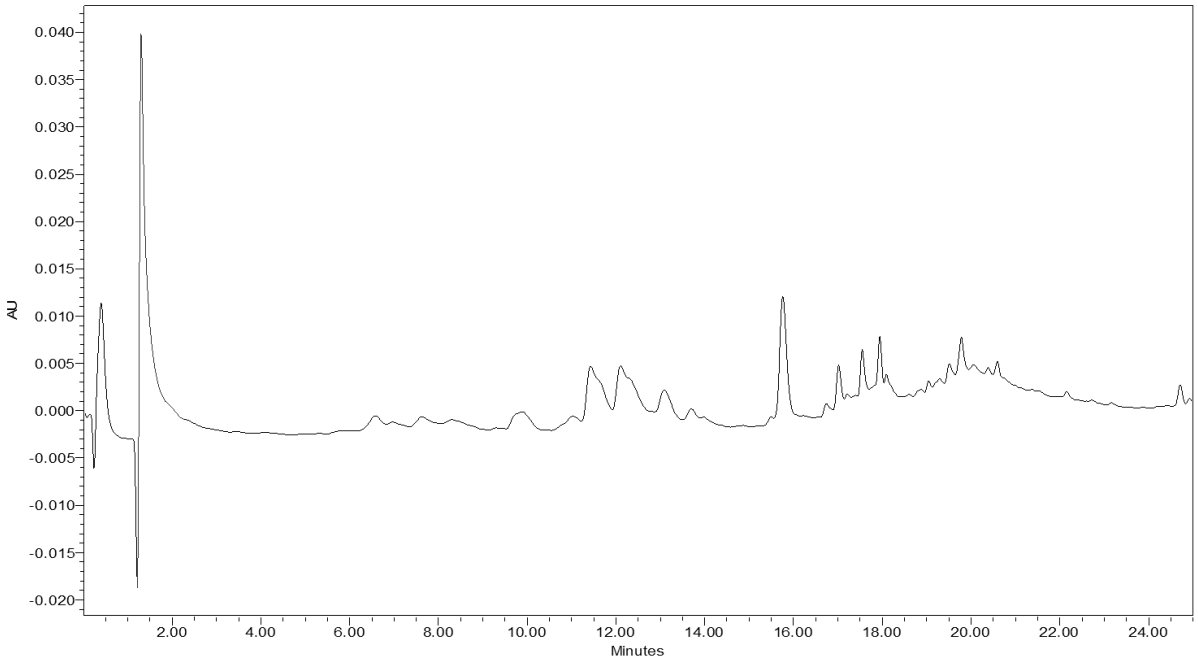
R-FSC-G2 Free 320nm



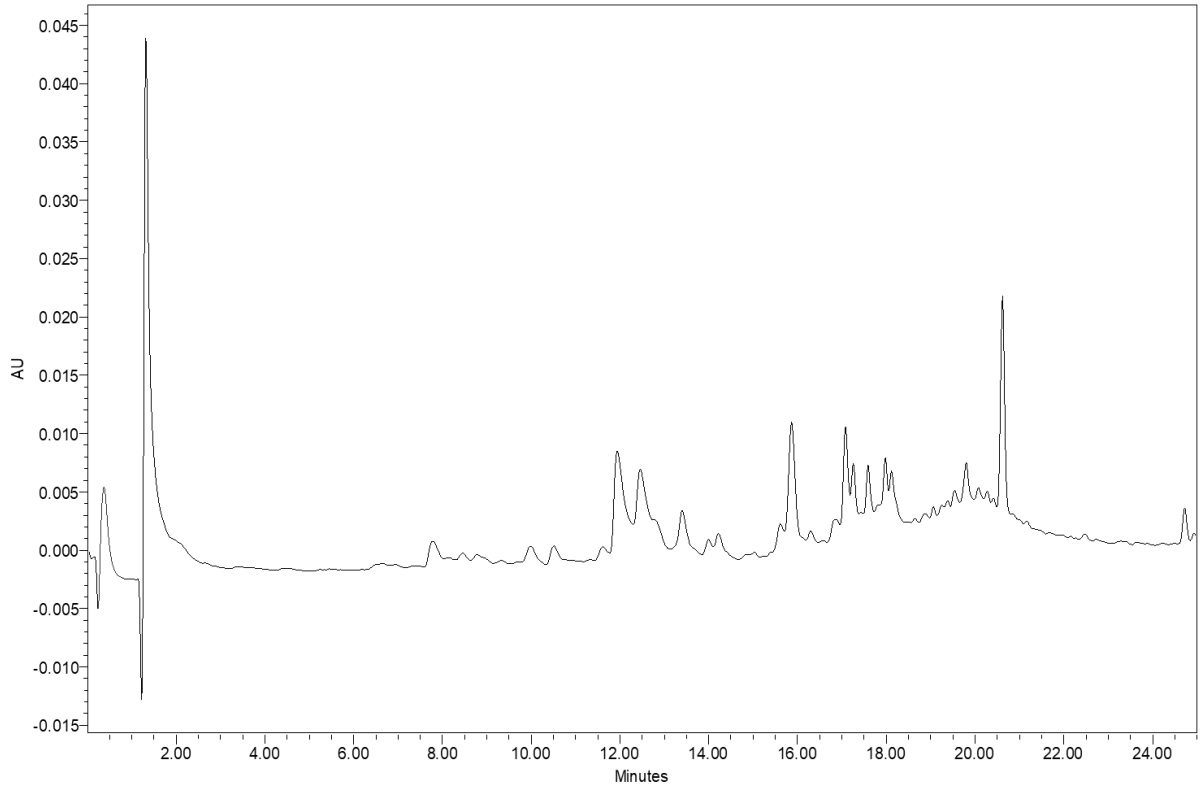
R-FSC-I1 FREE 320nm



R-pH-G1 FREE 320nm

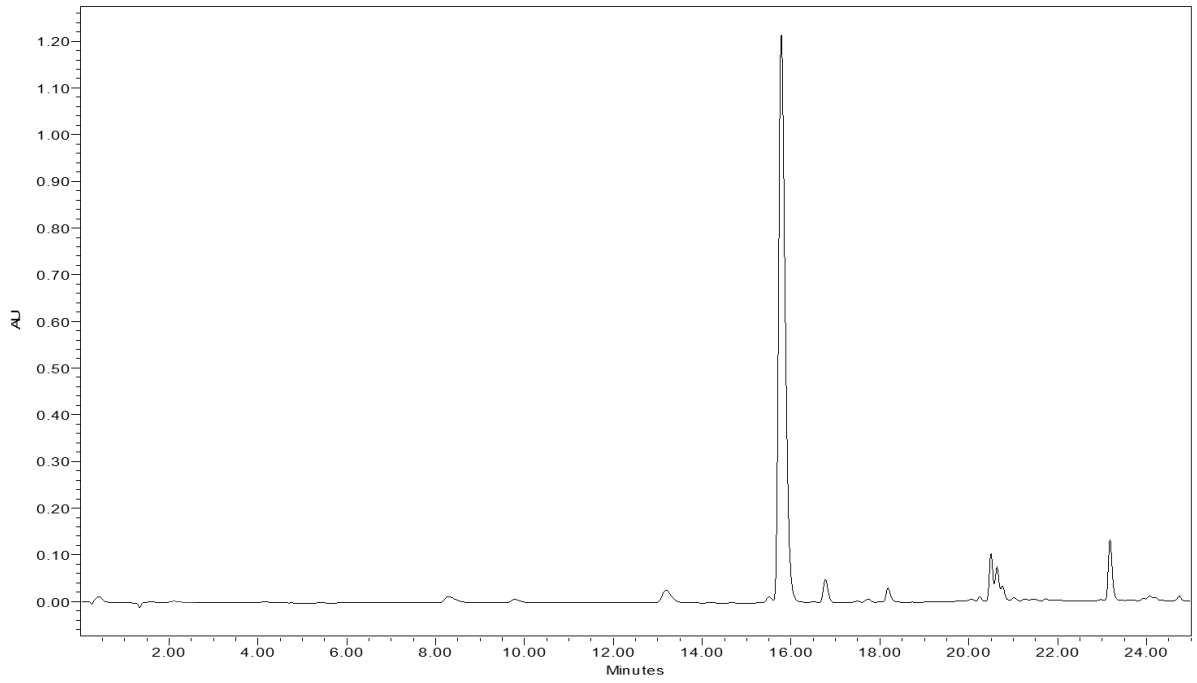


R-pH-I1 FREE 320nm

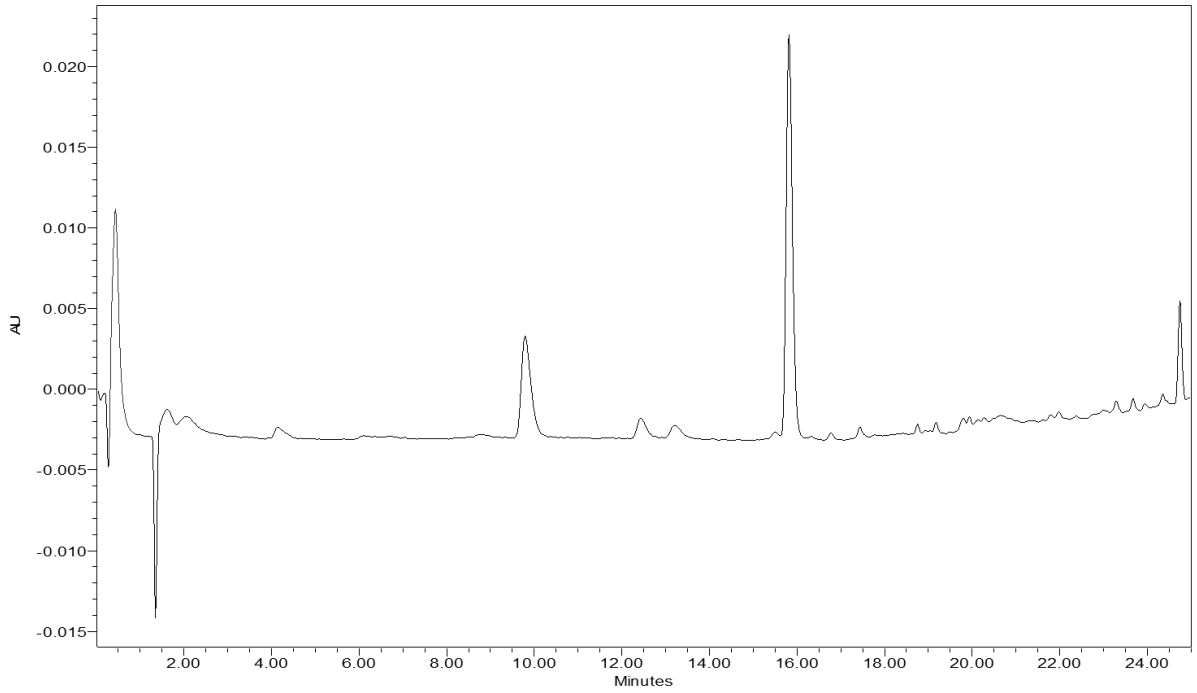


BOUND EXTRACTS

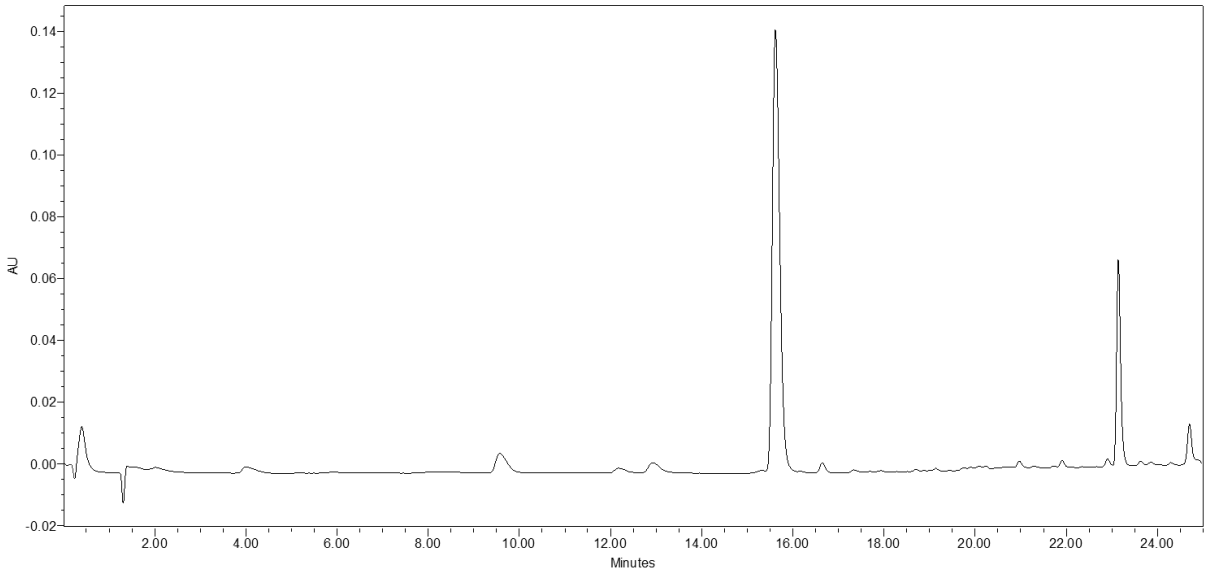
4_P2G Bound 320nm



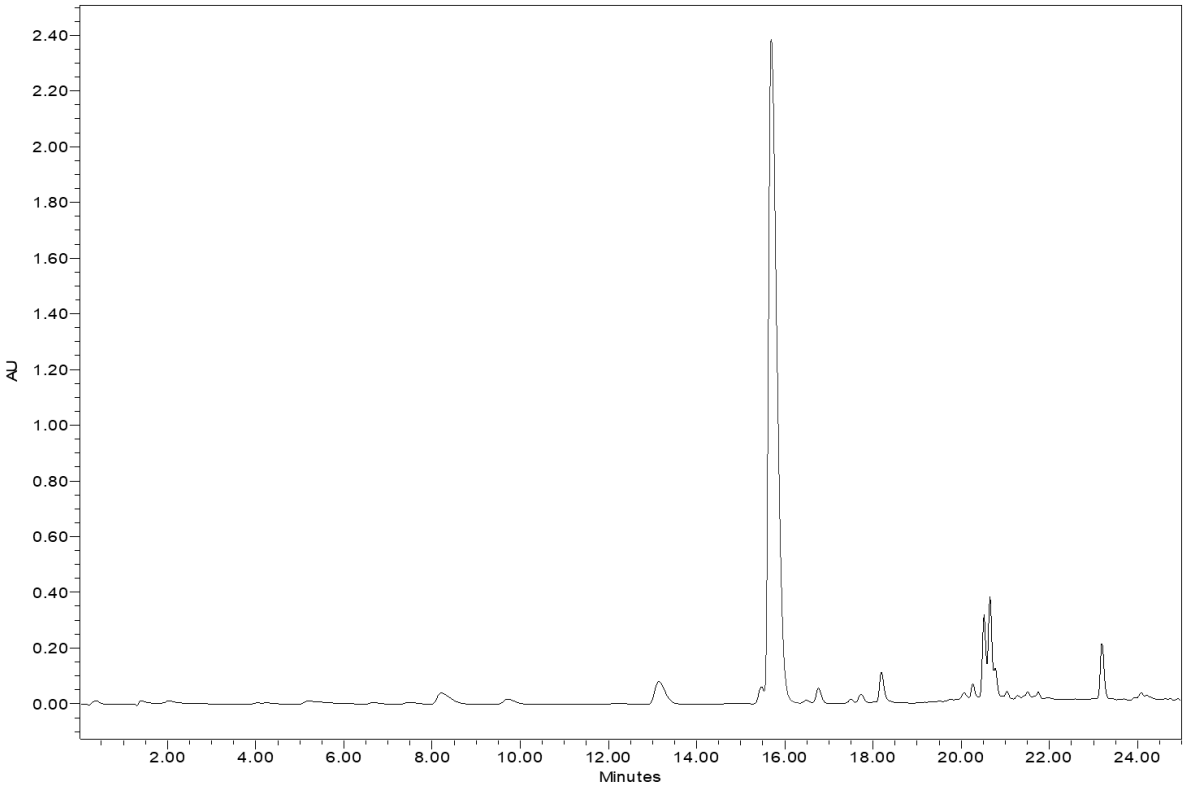
p-pH-G2 BOUND 320nm



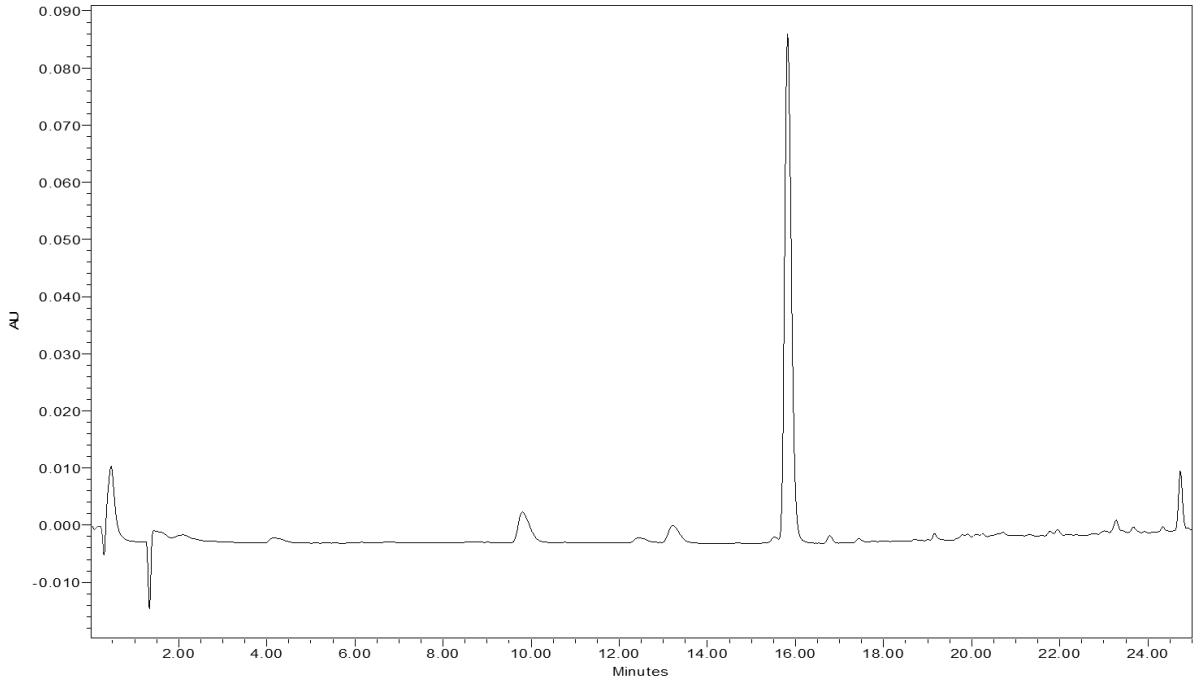
46_P-FSC-G1 Bound 320nm



P3I Bound 320nm



1_R2G Bound 320 nm



R1I Bound 320 nm