Evaluation of Physical and Antioxidant Properties of Pigmented Barley Tortillas

By

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ABSTRACT

Barley contains bioactive compounds like β-glucan and polyphenols that promote health. Food processing, while necessary, can change barley's physical properties and adversely affect polyphenols' antioxidant activity through heat and the food matrix. Thus, identifying compounds post-production, including new chemical structures, is essential. Five barley genotypes (CDC Rattan, CDC McGwire, Roseland, HB21147, and HB21148) produced tortillas at varying pH levels (1, 3, 5, 7). Their texture and physical properties were assessed. Identification and quantification were achieved through high-performance liquid chromatography (HPLC) with standards. Assays on the acidified ethanol extract measured the total phenolic and anthocyanin content, along with antioxidant properties, which were evaluated via in vitro and ex vivo methods. CDC Rattan tortillas decreased in thickness significantly between pH 1 and 7. At pH 5, extensibility was lower, with the hardest texture across genotypes. HB21148 recorded the highest total phenolic (14.0±1.2 mg gallic acid equivalent/g) and total anthocyanin content (313.9±33.4 cyanidin-3-glucoside equivalent/kg) among raw forms. Catechin was most concentrated in HB21148, followed by HB21147. Roseland exhibited the highest trans-cinnamic acid (13.5±0.5 mg/kg). Gentisic acid appeared only at pH 5 in genotypes with high β-glucan, HB21147 and CDC Rattan. Antioxidant activities were similar at pH 3 and 7 per the FRAP assay. HB21147 and Roseland showed parallel activity across pH levels with the ABTS method. Caco-2 cell tests found CDC Rattan enriched with anthocyanins resulted in significantly higher CAA (cellular antioxidant activity) than HB21148 at 200 µg/ml. The study suggests pH 3 optimizes antioxidant properties with minimal texture impact, potentially through new compounds involving β-glucan and polyphenols. These insights may aid in developing food products that integrate natural bioactive compounds for health benefits.

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LIST OF ABBREVIATIONS

AA	Antioxidant activity
AAE	Anthocyanin-rich aqueous extract
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
ACE	Anthocyanin crude extract
BQL	Below quantification limit
CAA	Cellular antioxidant activity
CDC	Crop Development Centre
CUPRAC	Cupric Ion Reducing Antioxidant Capacity
Cya-3-G	Cyanidin 3 glucoside
DMEM	Dulbeco's modified eagle medium
DPPH	2,2-diphenyl-1 picrylhydrazyl
DW	Dry weight
EDTA	Ethylene diamine tetra acetic acid
FRAP	Ferric reducing antioxidant power
FTIR	Fourier Transform Infrared Spectroscopy
GAE	Gallic acid equivalent
HAT	Hydrogen atom transfer
HB	Hull-less barley
HPLC	High-performance liquid chromatography
LDL	Low-density lipoprotein
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
NS	Not significant

NR	Not reported
OECD	Organization for Economic Co-operation and Development
ORAC	Oxygen Radical Absorbance Capacity
MG/KG	Parts per million
ROS	Reactive oxygen species
SET	Singal electron transfer
TAC	Total anthocyanin content
TPC	Total phenolic content
Tx eq	Trolox equivalent

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CHAPTER 1: **INTRODUCTION and LITERATURE REVIEW**

1.1. Introduction

Barley is the fourth most consumed grain worldwide (OECD-FAO, 2021). It is primarily used for animal feed (~80%) and malting production (~20%), with only approximately 2% allocated for direct human consumption (Government of Canada, 2019; Idehen et al., 2017). Barley contains dietary fibres (mainly β-glucan), vitamins, minerals, and phytochemicals.

According to Health Canada (2012), a health claim allows food products containing barley to state that consuming 3g of barley β-glucan per day can help reduce cholesterol levels, a known risk factor for heart disease. This claim permits the inclusion of barley in food products after processing, as long as the β-glucan content is maintained, without requiring extraction. The health claim states that barley β-glucan has better health benefits than oat β-glucan. Numerous reactions occur during food processing, including browning reactions such as Maillard and non-enzymatic browning. These reactions can influence the oxidation of polyphenols, potentially increasing or decreasing their antioxidant activity, physicochemical properties, and the colour of the product (Manzocco et al., 2020). Additionally, dietary fibre in baked goods plays a crucial role in determining texture. For instance, adding 2.5% barley β-glucan to bread significantly resulted in a denser texture (Brennan & Cleary, 2007). Conversely, in tortillas, texture alterations become pronounced when the concentration of β-glucan exceeds 4%, leading to a firmer product as well (Jurkaninová et al., 2024; Sánchez-Madrigal et al., 2015).

Barley also contains phytochemicals, considered secondary plant metabolites that positively affect human health by reducing the risk of cardiovascular and chronic diseases, such as cancer (Idehen et al., 2017; Martínez et al., 2018). Among the polyphenol subclasses, anthocyanins are the most studied in barley (Idehen et al., 2017). Anthocyanins provide health

benefits, including antihypertension (Ed Nignpense et al., 2022) and antidiabetic, anti-inflammatory, and anti-obesity effects (Delgado-Povedano et al., 2021). Previous studies reported that polyphenols and anthocyanins, in particular, are highly sensitive to various environmental factors, such as pH, temperature, oxygen, sugars, and metals, which can cause them to lose their chemical properties and also display different colours (Campos-Vega & Oomah, 2013; Ed Nignpense et al., 2022; Markakis, 1982; Zhang et al., 2017).

After processing, it is necessary to identify and quantify the bioactive compounds as they may undergo changes. Apart from the known methods, high-pressure liquid chromatography (HPLC) and mass spectrophotometer (MS), Fourier transform infrared spectroscopy (FT-IR) can give more information about the chemical changes. Several applications of FT-IR include identifying compounds and detecting modifications to chemical bonds (Gui et al., 2023). Polyphenols, anthocyanins, and dietary fibre are ideal candidates for finding changes in the chemical structure due to oxygen in their structure (Shi et al., 2022). Oxygen and double bonds of carbon in molecules are excited during infrared (IR) projection, which gives essential information on the changes within their structures.

Another aspect of the functional properties of phytochemicals is their antioxidant activity. A wide range of antioxidant activity assays differ based on the mechanism, either *in vitro* or *ex vivo* (Sadeer et al., 2020). Using human cells for antioxidant activity assays can be expensive, lengthy, and challenging (Lea, 2015).

Polyphenols and anthocyanins, in particular, are sensitive to pH and the food matrix. Therefore, the literature on the effect of pH on food products is limited in terms of its understanding of the stability mechanism. Assessing the bioactive compounds and their antioxidant activity is critical for the future use of new grains after they have been processed. The current work aims to

investigate the effect of pH levels (1, 3, 5 and 7) on barley tortillas from the physical, antioxidant activity and bioactive compounds aspects.

1.2. Literature Review

1.2.1 Barley

Barley offers numerous health benefits, earning a health claim from Health Canada (2012). Similarly, the US FDA (2006) and the EU EFSA (2011) recognize that consuming barley can lower the risk of heart disease and help manage postprandial blood glucose levels. Barley is also rich in minerals, vitamin B, and phenolic compounds and low in fat (Badea &Wijekoon, 2021), making its nutritional profile a valuable asset in preventing chronic illnesses. It is important to note that barley varieties are developed according to the end usage: malting, food and general purpose.

Barley processing begins with three primary forms: hulled, hull-less, and pearled grains. Hulled barley has a strong connection between the hull and the kernel, whereas hull-less barley has a loose connection. To make barley edible, dehulling and/or pearling is required (Izydorczyk & Dexter, 2016). The pearling process involves removing 30% of the grain's weight, removing the outer layers and producing pearled barley (Figure 1.1). However, pearling can reduce anthocyanin

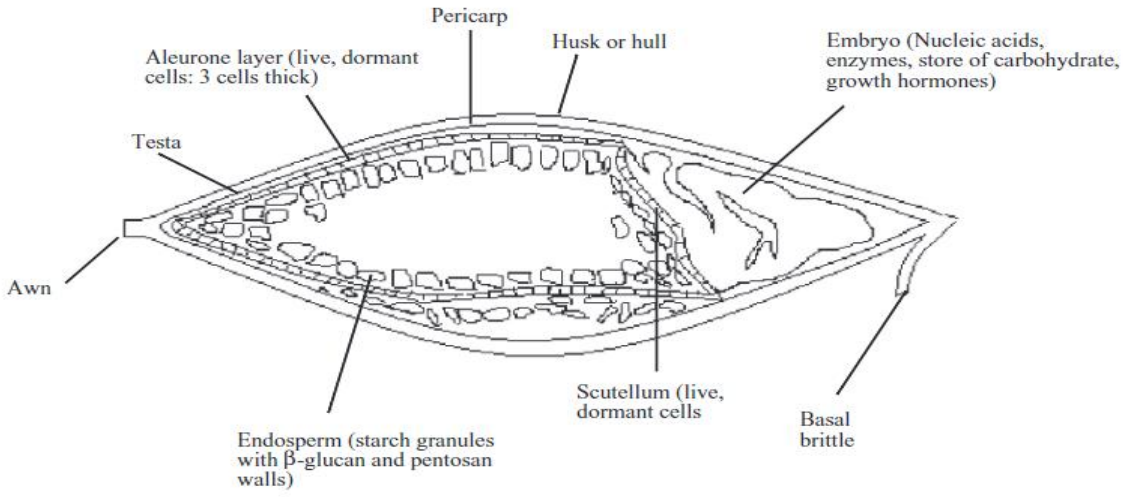

Figure 1.1. Barley grain structure (Buglass, 2011, with permission).

content, as these compounds are in the pericarp and aleurone layers (Idehen et al., 2017). Milling can also lead to the loss of beneficial compounds, which are often concentrated in the hull fractions and outer layers of the grain, including pericarp and aleurone layers (Izydorczyk & Dexter, 2016). Some barley varieties are specifically designed for flour production and streamlining processing (Badea et al., 2017). Barley is versatile and can be used in various products, including flatbread, noodles, pasta, muffins and tortillas (Baik, 2014). Baik (2014) suggests that hull-less or naked barley has greater potential for use in food products due to its reduced processing requirements. However, the presence of β-glucans may affect texture, as discussed later. Research indicates that consuming barley foods rich in β-glucans can slow glucose absorption and lower the glycemic index. The natural bioactive compounds in barley are believed to play a crucial role in this process (Aldughpassi, Wolever, & Abdel-Aal, 2016).

Furthermore, the pH of food products and various processing steps affect anthocyanins and other compounds, including polyphenols and β-glucans, as well as their interactions. Therefore, investigating these relationships and the effects of individual compounds is crucial.

1.2.2 Polyphenols

Phytochemicals, also known as secondary plant metabolites, have been shown to positively influence human health by potentially reducing the risk of cardiovascular and chronic diseases, including cancer (Huang, Xiao, Burton-Freeman, & Edirisinghe, 2016). Polyphenols are present in cereals containing polyphenols, which can be either free or bound to the cell walls. To analyze these polyphenols, they must be extracted under alkaline conditions (Bonoli, Verardo, Marconi, & Caboni, 2004). While acetone and water extracts yield higher total phenolic content, the acid extraction method shows the highest antioxidant activity.

Polyphenols are sensitive to processing conditions; however, their stability primarily depends on their source. Generally, they degrade thermally in a predictable, first-order pattern, following Arrhenius-like behaviour (Debelo, Li, & Ferruzzi, 2020). Notably, temperature is not the only factor affecting polyphenols. Heat, combined with proteins and sugars, can enhance interactions with polyphenols, influencing their stability (Gurley, Tonsing-Carter, Thomas, & Fifer, 2018; Hurst et al., 2011).

Brglez Mojzer et al. (2016) revealed that polyphenols have limited bioavailability and are rapidly metabolized. This suggests that using various encapsulation methods could overcome these limitations. Additionally, Angulo-López et al. (2022) found that β-glucans play a role in stabilizing polyphenols, which could further enhance their effectiveness.

The studies mentioned earlier mainly focus on how added ingredients interact. Still, they do not explore how the natural compounds within those ingredients interact, such as β-glucans, polyphenols, sugars, proteins, and minerals. Examining food products with simple ingredients and minimal processing is crucial to better understand these interactions. Each processing step can significantly impact the release of phenols, affecting the total phenolic content (Bonoli, Verardo, Marconi, & Caboni, 2004).

Polyphenols are a large and diverse group of compounds, with over 8,000 identified. They are divided into four main categories: phenolic compounds, flavonoids, stilbenes, and lignans (Pandey & Rizvi, 2009). The flavonoid category is subdivided into six classes, each sharing a basic structure with oxygen in the C ring. These subclasses include flavonols, flavanones, flavanols, anthocyanins, and isoflavones.

As mentioned earlier, anthocyanins are a subclass of polyphenols, which means they share similar stability and degradation patterns. Several polyphenols have been identified, and their structures are illustrated in Figure 1.2.

Polyphenols and anthocyanins are crucial for plant pigmentation (Brouillard, 1982; Pandey & Rizvi, 2009). This pigmentation serves two important purposes: it gives plants their colour and acts as an antioxidant shield, protecting them from UV damage. Anthocyanins, a subgroup of polyphenols, have been extensively studied in this context.

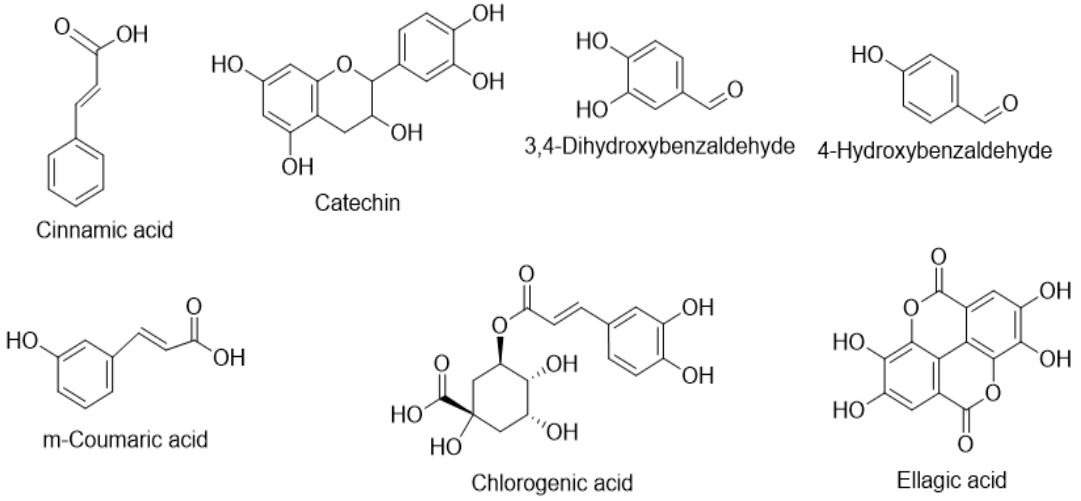

Figure 1.2 Different polyphenol structures

1.2.3 Stability and Structure of Anthocyanins

Anthocyanins have a distinct chemical structure, typically consisting of a 3,5,7,4'-tetrahydroxylavylium cation. This structure features a benzene ring (B ring) linked to a five-carbon ring (C ring) with cation oxygen, which shares two carbons with another ring (A ring) (Brouillard, 1982) (Figure 1.3A). There are six main anthocyanins: pelargonidin, cyanidin, peonidin, delphinidin, malvidin, and petunidin. These differ in the number and location of hydroxyl groups

on the B ring and the presence of methoxylated residues (Brouillard, 1982; Gamel et al., 2023) (Fig 1.3B and 1.3C, respectively).

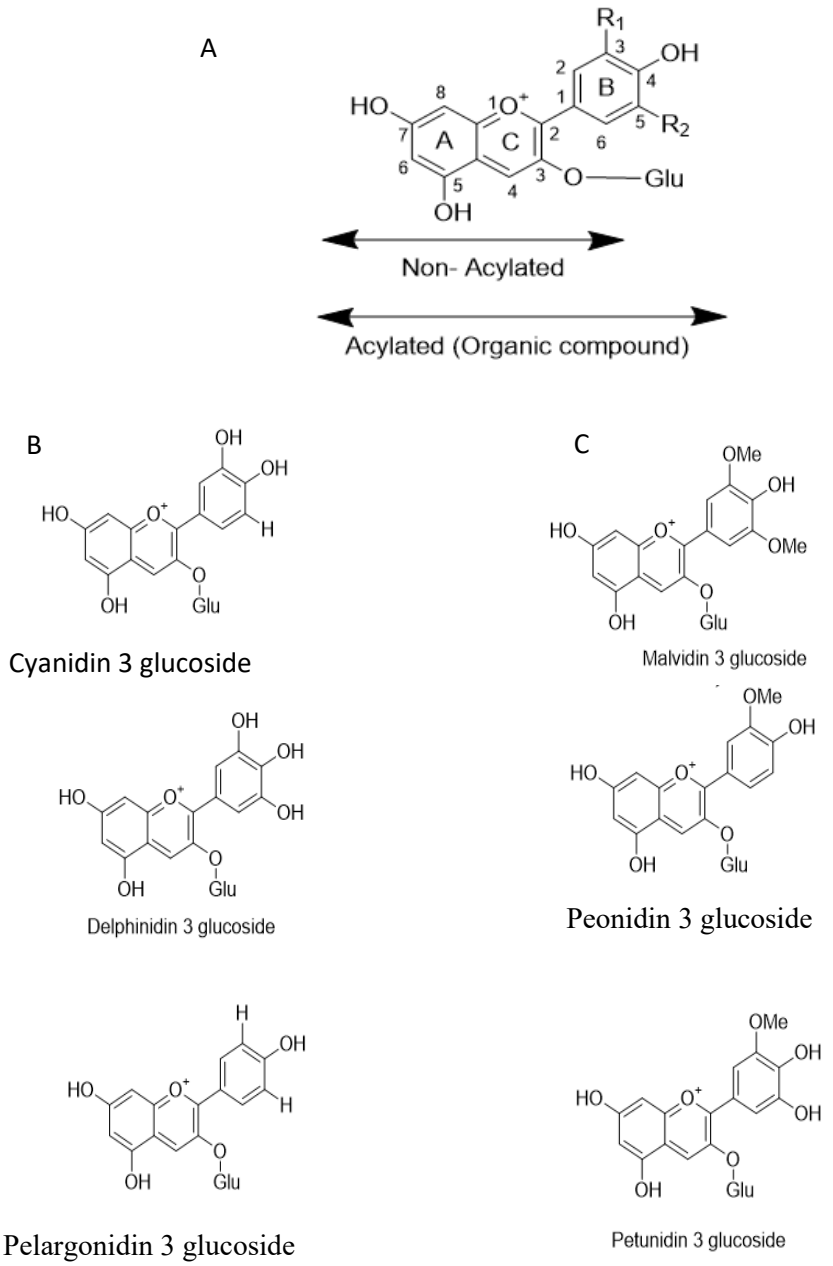

Figure 1.3. Anthocyanin backbone structure (A), Major unmethylated anthocyanins (B), Major methylated anthocyanins (C).

Anthocyanins retain their chemical structure and colour at pH 1, existing as flavylium cations (Yan et al., 2023). However, as the pH increases (2-4), a hydroxyl group reacts with the C ring (position 2'), causing the anthocyanin to lose its stability (Brouillard, 1982). The flavylium cation loses its proton, forming two quinoidal blue bases with two successive pK_a values (Chen et al., 2023). Colourless forms emerge at pH 5-6, including carbinol pseudobase and chalone (Enaru et al., 2021). These forms can co-exist with the flavylium cation at pH 4-6 (Enaru et al., 2021). At even higher pH levels, anthocyanins degrade and form a blue-ionized quinone base, influenced by their substituent groups (Enaru et al., 2021; Yan et al., 2023) (Figure 1.4).

Several factors, including high pH, oxygen, and heat, are known to accelerate the degradation of anthocyanins (Brouillard, 1982; Enaru et al., 2021; Markakis, 1982). According to Yan et al. (2023), exposure to oxygen and heat can cause anthocyanins to undergo hydrolysis or deglycosylation, resulting in the formation of chalcones and subsequent degradation into phenolic acids. However, co-pigmentation can stabilize anthocyanins (Chen et al., 2022). Co-pigmentation is unique to anthocyanins and enhances their stability by forming molecular or intermolecular interactions with colourless compounds rich in π electrons. These interactions strengthen the stability of flavylium ions and intensify their colour (Castañeda-Ovando et al., 2009; Enaru et al., 2021). Co-pigmentation can involve various substances, including flavonoids, alkaloids, amino acids, organic acids, nucleotides, polysaccharides, metals, and even other anthocyanins (Castañeda-Ovando et al., 2009). Notably, these interactions are pH-dependent, and lower pH values can increase maximum absorption by 5-20 nm and enhance antioxidant activity (Castañeda-Ovando et al., 2009; Enaru et al., 2021; Yan et al., 2023).

Lowering the pH level can help stabilize anthocyanins when exposed to heat, as Enaru et al. (2021) suggested. Maintaining a low pH, around 1-2, can increase and protect anthocyanin

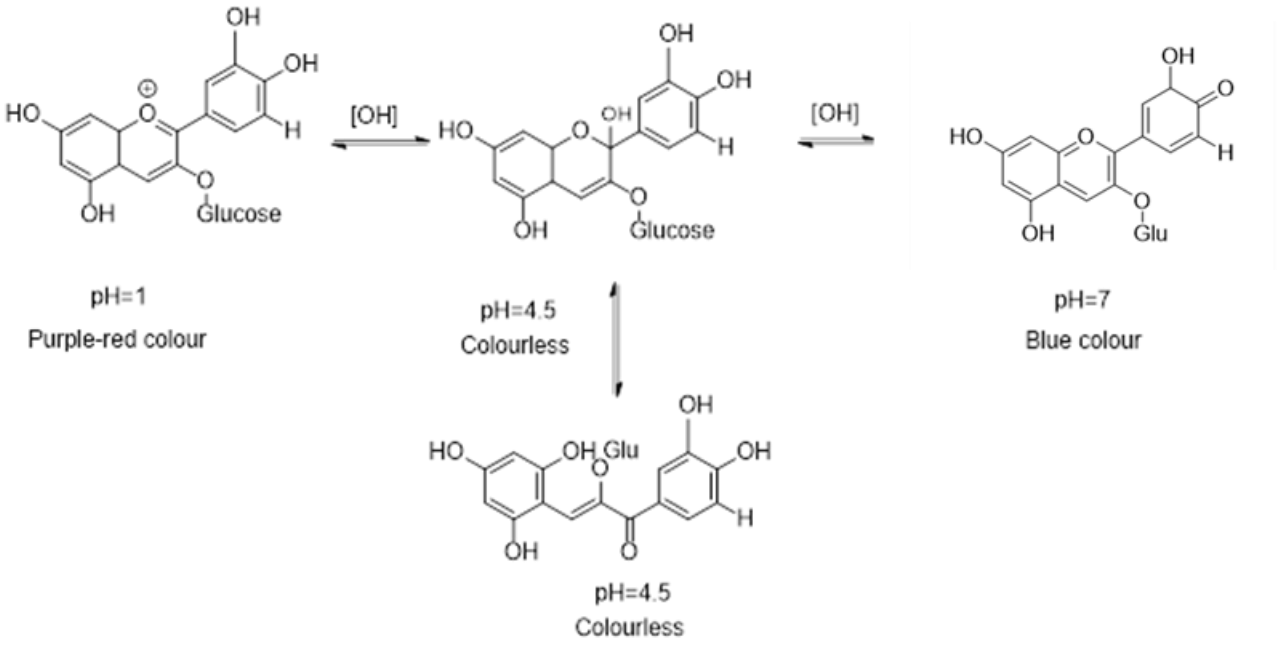

Figure 1.4. Effect of pH on Cyanidin-3-glucoside, where Glu is glucose.

levels in food (Gamel et al., 2023). Earlier research by Li et al. (2010) supported this idea, finding that lower pH levels correlated with higher total anthocyanin content in baked cookies.

1.2.4 Fourier Transform Infrared Spectroscopy (FTIR)

Table 1.1 summarizes existing literature on interactions between various compounds and anthocyanins using FTIR. Low pH has been shown to enhance the stability of anthocyanin. However, it may also lead to the formation of complexes with other compounds, such as β-cyclodextrin (βCD) and cycloamylose (CA) (Jung, Joo, Rho, & Kim, 2020). FTIR analysis revealed that black rice extract forms complexes with CA and βCD, as indicated by the decreased characteristic peaks at 1330-1633 cm^{-1}, suggesting a reduction in C=C stretching vibrations (Jung, Joo, Rho, & Kim, 2020). Improved stability and antioxidant activity of anthocyanins were observed at pH 2 after heating the solution at 95°C for 24 hours. Earlier studies on βCD and β-glucan have demonstrated that encapsulation protects antioxidant compounds, likely due to the

presence of a typical band at 1700 cm^{-1} (Ahmad, Ashraf, Gani, & Gani, 2018). Moreover, combining bacterial cellulose with anthocyanins resulted in a change in bond intensity at 1703 cm^{-1}, indicating the formation of new intermolecular hydrogen bonds (Shi, Ji, Zhang, Jia, & Yang, 2022). In a study investigating the degradation of Cya-3-Glu under constant heating, Dong, Li, Zhang, Liu, & Ren (2022) found that bonds in this range began to weaken at 330°C. However, this temperature was only reached after nearly 30 minutes, suggesting relatively stable and heat-resistant bonds.

A significant band at 1640 cm^{-1} has been observed, with increased absorption intensity at pH 4 in pigmented maize (Bhushan et al., 2023). Additionally, this band shifts from 1646 cm^{-1} to 1650 cm^{-1} due to interactions between myofibrillar proteins (MP) and anthocyanins from black rice (Chen et al., 2023). The interaction between MP and anthocyanins can improve the stability of films, leading to the development of enhanced packaging materials. Furthermore, the characteristic $C=O^{+}$ band stretching of the flavylium cation at 1720 cm^{-1} showed decreased intensity at both pH 5 and pH 7 when Cya-3-G was combined with potato starch (Li et al., 2022). The transition of the bond from $-C=O^{+}$ to -O- suggests that the stabilization of anthocyanins is not solely dependent on hydrogen bonds or covalent interactions, as the presence of NaCl did not enhance stability (Li et al., 2022).

Gui et al. (2023) investigated how pH changes affect the interaction between Cya-3-G and lysozyme. They found significant changes in the amide I structure at pH 3, particularly in the C=O bond. At pH 7.4, two peaks were observed, whereas at pH 3, only one peak at 1641 cm^{-1} was observed. Additionally, the amide II peaks shifted from 1546 cm^{-1} to 1537 cm^{-1}, indicating that anthocyanins induce structural changes in lysozyme. Notably, these changes cannot be attributed to hydrogen bonds as they do not alter the chemical structure of molecules (Marechal, 2007).

Resende, Oliveira, & Franca (2020) compared different Jabuticaba peel varieties rich in anthocyanins. They found that anthocyanin levels decrease as insoluble dietary fibre increases, suggesting that the antioxidant compounds present in different Jabuticaba varieties may affect human health differently. The stability of anthocyanins is likely due to hydrogen bonding, a concept supported by Pimentel & McClellan (1960) and later by Marechal (2007). Hydrogen bonds can form within a single molecule (intramolecular) and or between different molecules (intermolecular). These bonds require an acidic group (a proton donor) and a basic group (an electron donor). Intramolecular hydrogen bonds, which form within a single molecule, also known as chelation, occur within a molecule. In contrast, intermolecular hydrogen bonds occur between different molecules, commonly found in phenols and polyhydroxy materials, creating rings and three-dimensional networks (Pimentel & McClellan, 1960).

According to Marechal (2009), cellulose has a dense network of hydrogen bonds, which makes it poorly soluble in water. This low solubility also makes cellulose difficult to hydrolyze and, as a result, challenging for humans to digest. The process involves the β-linkages between monosaccharides, which play a crucial role. Proton transfer is essential to the hydrogen bond mechanism. When protons are exposed to visible or UV photons, they become electronically excited and undergo proton transfer, known as excited-state proton transfer (ESPT). Compounds involved in this process are known as photoacids. In phenols or naphthols, an O-H group can replace a C-H bond. The O-H group becomes excited when exposed to visible or UV light, transferring electrons to the aromatic ring.

Changes in the hydrogen bond formed by the O-H group in phenols can lead to rapid proton transfer, causing the anion to behave like a strong acid. According to Marechal (2009), anthocyanins are considered "superphotoacids" due to their exceptional ability to undergo excited-

state proton transfer. This property makes them effective protective agents in plants, pigmented grains, and cereals.

In summary, low pH significantly influences anthocyanins by facilitating the formation of new structures with various compounds. Moreover, dietary fibres can potentially enhance anthocyanin content and antioxidant activity. When employing FTIR analysis, it is recommended to thoroughly examine peaks in the 1700-1600 cm^{-1} range, along with the characteristic features, to determine the impact of pH on anthocyanins. Since hydrogen bonds rely on acid-base interactions, pH changes can significantly impact the stability mechanism. While numerous studies have investigated the effects of pH or different compounds on anthocyanins and phenolic acids, there is a notable lack of research exploring the potential interactions between anthocyanins and other compounds within samples, such as those found in pigmented grains and cereals.

1.2.5 Evaluation of Anthocyanins

Anthocyanins exhibit distinctive characteristics and vary in prevalence across different plants. Cyanidin, the most abundant anthocyanin backbone, produces a red-purple pigment (Enaru et al., 2021). Pelargonidin is red in its free form, appearing orange in flowers and red in fruits. Environmental factors influence delphinine, which can appear reddish-purple in grains or blue in flowers. Petunidin, a water-soluble methylated anthocyanin, displays dark red or purple pigments and is commonly found in purple flowers. The remaining anthocyanins, peonidin and malvidin, account for 7% of all anthocyanins (Enaru et al., 2021). Peonidin, similar to petunidin, primarily occurs in berries, grapes, and red wine, producing a magenta pigment. Malvidin, an O-methylated anthocyanin, regulates blue coloration in specific flowers, although its colour is purple (Enaru et al., 2021).

Evaluation is necessary due to variations in anthocyanin concentration and composition in plants and grains. Three methods exist for identifying and quantifying anthocyanins: High-Performance Liquid Chromatography (HPLC), Mass Spectrometry (MS), and Nuclear Magnetic Resonance (NMR).

HPLC is the most common method for detecting chemical compounds, with well-established conditions for identifying anthocyanins (Avula et al., 2023). However, HPLC requires standards of the expected anthocyanins, limiting identification and quantification to these specific compounds in samples (Yan et al., 2023).

Table 1.1. FT-IR Information on Anthocyanins with Other Compounds

Material	Band stretching	Peak wavenumber (cm^{-1})	Treatment effect	Explanation	Reference
Saffron anthocyanins and β-glucan and β CD	C=C (aromatic ring)	1700	The band in this region was affected	Encapsulation of anthocyanins with fibres caused a transformation in their molecular structure.	Ahmad, Ashraf, Gani, & Gani. (2018)
	β-glycosidic anomeric bonds	890	β-configuration		
Jabuticaba peels	Esterified and non-esterified carboxyl groups	1760-1650	Soluble DF (pectin)		Resende, Oliveira, & Franca (2020)
	Asymmetric stretching modes vibration of methyl esters	1440			
	Ring vibrations	1022, 953			
	Phenolic compounds	3600-3200,1410-1320,1260-1180	Interaction of angular deformation of O–H and C–O stretching. The 3600-3200 cm^{-1} can be a characteristic area of saccharides.		
C-3G degradation in nitrogen	H_2O	3500-3900	Gradually weakened at 240°C	Pyrolysis of C-3-G started at 200°C, and new compounds formed. From 300°C, CO_2 was the main compound released.	Dong, Li, Zhang, Liu, & Ren. (2022)
	C-H	3000-2750			
	CO_2	2400-2300	Slightly weakened and then increased at 330°C		
	C=O/ C=C (aldehydes, carboxylic acids, and olefins)	1800-1500	It began to weaken at 330°C		
	C-O-C (bending vibration of the aromatic compound)	1150-890	Weakened at 420°C		

Table 1.1 cont.

Material	Band stretching	Peak wavenumber (cm^{-1})	Treatment effect	Explanation	Reference
Cya-3-G standard	C-H (bending vibration of the aromatic compound)	670		Pyrolysis of Cya-3-G started at 200°C, and new compounds formed. From 300°C, CO_2 was the main compound released.	Dong, Li, Zhang, Liu, & Ren. (2022)
Potato starch and Cya-3-G	$C=O^+$	1720	Structural feature of anthocyanins at pH 3 in the flavylium cation.		Li et al. (2022)
		1720, 1442	Disappeared or weakened at pH 5 and pH 7	Transformation of -C=O to -O-	
	C-O glycosyl group	1326-1016			
Cya-3-G and bacterial cellulose	C=C (aromatic ring)	1703	The bond intensity became more intense.	The Cya-3-G was successfully immobilized in the bacterial cellulose.	Shi, Ji, Zhang, Jia, & Yang. (2022)
Colour maize	Benzene ring	1500-2000			Bhushan et al. (2023)
	Ring vibrations	1330, 1530, 1590 and 1640	It had the strongest signal at pH4		
	C-OH	1240	Characteristic feature of acylated anthocyanins		
Myofibrillar proteins (MP)- black rice	C=O	1646	Shifted from 1646 to 1650	These interactions altered the structure of polypeptide chains in the MP edible film.	Chen et al. (2023)
Cya-3-G and lysozyme	N-H (non-hydrogen bonded) O-H (free stretching)	3270	The peak shifted to 3274 at pH 3 and from 3286 cm-1 to 3288 cm-1 at pH 7.4 in the lysozyme-C-3-G complex.	Part of the hydrogen bond in the lysozyme was destroyed after the binding.	Gui et al. (2023)

MS determines molecular structure by analyzing the ratio of mass to charge in ionized molecules within a magnetic field and vacuum. However, this method is destructive. The resulting data is compared to digital libraries (Bjerrum, 2015).

In contrast, NMR spectroscopy is a non-destructive technique that measures changes in atomic nuclei, mainly hydrogen and carbon, when exposed to a strong magnetic field (Avula et al., 2023; Yan et al., 2023).

1.2.6 Anthocyanin Composition in Pigmented Barley

Cyanidin-3-glucoside is the predominant anthocyanin compound in purple barley, as consistently detected in all referenced studies (Table 1.2). This compound is the standard for calculating total anthocyanin content (AOAC SMPR 2011.011; Yan et al., 2023; Zhang et al., 2020). The second and third most frequently detected compounds are pelargonidin-3-glucoside and peonidin-3-glucoside, respectively. Zhang et al. (2020, 2021) employed Liquid Chromatography-Mass Spectrometry (LC-MS) to detect similar compounds in purple barley. However, they exclusively reported certain compounds, including cyanidin acetyl galactoside and cyanidin-3-(6″-succinyl) glucoside and two isomers (Zhang et al., 2021). Their earlier study identified different isomers, such as cyanidin malonyl glucoside and peonidin glucoside. Kohyama et al. (2008) used NMR to analyze purple barley, discovering a new compound, cyanidin malonyl glucoside and three reported isomers. Although NMR effectively identifies new compounds, LC-MS uniquely detected eleven compounds (Ed Nignpense et al., 2022; Lee et al., 2012; Zhang et al., 2020, 2021; Zhang et al., 2017). Lee et al. (2012) successfully identified 60% of the total anthocyanin content, while Bellido & Beta (2009) reported twelve unidentified peaks when using HPLC.

Table 1.2. Anthocyanin Composition in Purple Barley

Anthocyanidin backbone	Compound	Reference
Cyanidin	Cyanidin-3-glucoside	Kim 2007, Kohyama 2008, Bellido 2009, Lee 2012, Zhang 2017, Zhang 2020, Dang 2022, Ed Nignpense 2022, Jin 2022
	Cyanidin acetyl galactoside	Zhang 2020, Zhang 2021
	Cyanidin di-glucoside	Zhang 2020
	Cyanidin malonyl glucoside	Kohyama 2008, Zhang 2020, Zhang 2021
	Cyanidin-3-(6″-succinyl) glucoside	Lee 2012, Zhang 2021, Jin 2022
	Cyanidin-3-galactoside	Zhang 2020
	Cyanidin-3-rutinoside	Zhang 2017
Delphinidin	Delphinidin-3-glucoside	Bellido 2009, Kim 2007, Zhang 2017, Dang 2022
Malvidin	Malvidin 3-(6"-acetylglucoside)	Ed Nignpense 2022
	Malvidin-3-glucoside	Kim 2007, Zhang 2017, Dang 2022, Jin 2022
Pelargonidin	Pelargonin	Zhang 2017
	Pelargonidin-3-glucoside	Kim 2007, Bellido 2009, Zhang 2020, Zhang 2021, Dang 2022, Jin 2022, Lee 2022
Peonidin	Peonidin acetyl galactoside	Zhang 2021
	Peonidin glucoside	Zhang 2020
	Peonidin-3-(6″-succinyl) glucoside	Lee 2012
	Peonidin-3-glucoside	Kim 2007, Bellido 2009, Lee 2012, Zhang 2017, Lee 2022
Petunidin	Petunidin-3-glucoside	Bellido 2009, Dang 2022, Jin 2022

1.2.7.1 Antioxidant Activity

The chemical composition of different layers varies, influencing their antioxidant capacity. In the human body, oxidation occurs through the constant production of hydrogen peroxide (H_2O_2), a highly reactive oxygen species (ROS) that forms hydroxyl radicals ($OH^{\bullet}$). This process leads to lipids and low-density lipoprotein (LDL) oxidation, potentially damaging DNA. Oxidative processes like these contribute significantly to the development of diseases. As a result, researchers are continually seeking antioxidant-rich compounds in various food products (Kiokias et al., 2018).

1.2.7.2 Antioxidant Methods

Researchers primarily use two mechanisms to evaluate antioxidant activity: Hydrogen Atom Transfer (HAT) and Single Electron Transfer (SET) (Enaru et al., 2021). The Oxygen Radical Absorbance Capacity (ORAC) assay is a notable example of the HAT method. This widely used technique efficiently assesses the antioxidant activity of anthocyanins in various food types. ORAC measures fluorescence changes using a spectrophotometer to evaluate the rate of free radicals and the extent of inhibition by generating hydrogen atoms. This method requires a temperature of 37°C and a pH of 7.4 (Enaru et al., 2021).

Ferric-reducing Antioxidant Power (FRAP) and Diphenyl-1-Picrylhydrazyl ($DPPH^{\bullet}$) are examples of SET assays. FRAP is a colourimetric technique that measures the capability of antioxidants to convert Fe^{3+} to Fe^{2+}, forming a solid blue colour. This method requires a pH of 3.6 and a temperature of 37°C (Enaru et al., 2021). The $DPPH^{\bullet}$ assay is versatile, accommodating both solid and liquid samples, and assesses the ability of the free radical in the sample to react with $DPPH^{\bullet}$, reducing its violet colour.

The Cupric Ion Reducing Antioxidant Capacity (CUPRAC) is another spectrophotometric technique that evaluates the antioxidant capacity of a sample by measuring the reduction rate of

Cu^{2+} to Cu^{+} at pH 7 (Enaru et al., 2021). Although CUPRAC is a rapid method, anthocyanins may require additional treatment to enhance their reduction potential. In contrast, the 2,2-Azinobis-(3-Ethylbenozothiazoline-6-Sulfonate) radical cation ($ABTS^{\bullet+}$) assay uses spectrophotometry to measure changes in colour intensity, which occurs when the sample reacts with $ABTS^{\bullet}$. This reaction assesses the sample's antioxidant concentration and activity rate (Enaru et al., 2021). The ABTS assay is particularly useful for evaluating antioxidant activity based on the water solubility and pH levels of the compounds present in the sample, making it effective for analyzing both hydrophilic and lipophilic compounds.

1.2.7.3 Antioxidants in Pigmented Barley

Table 1.3 summarizes the antioxidant activity assays conducted on purple barley. Kim et al. (2007) used the $DPPH^{\bullet}$ assay to compare the antioxidant activity of hulled and hulless purple barley. They found that hulled grain had approximately 6% lower $DPPH^{\bullet}$ activity than hulless grain, which showed the lowest observed activity. Conversely, Bellido & Beta (2009) reported that the bran, which accounts for 10% of the grain, had an antioxidant capacity nearly four times greater than the whole grain in the ORAC assay. Lee et al. (2013) investigated the total anthocyanin content in various barley cultivars. They found that purple barley had significantly higher anthocyanin concentration, with the bran containing two to six times more anthocyanins than whole and pearled grains. Zhang et al. (2020) examined how different extraction methods affected the anthocyanin composition, stability, and antioxidant activity of purple highland barley bran. Their analysis used anthocyanin crude extracts (ACE) and anthocyanin-rich aqueous extracts (AAE). In the $ABTS^{\bullet+}$ assay, AAE showed antioxidant activity comparable to ascorbic acid, while ACE had a lower scavenging ability. The FRAP method revealed that increasing the concentration of both AAE and ACE enhanced antioxidant activity, with AAE being more than four times more

potent than ACE. Interestingly, $DPPH^{\bullet}$ activity was found to be concentration-independent. The antiradical activity of ACE increased systematically, but AEE remained significantly higher until approximately 300 µg/mL, after which the $DPPH^{\bullet}$ activity began to favour ACE. These findings are consistent with those reported by Bellido & Beta (2009).

Zhang et al. (2021) conducted a study on antioxidant activity, building upon their previous work (Zhang et al., 2020), focusing on different grain parts. As expected, the purified anthocyanin extracts exhibited antioxidant activity comparable to that of ascorbic acid, which served as the control. The purified extract demonstrated the highest activity levels in the $ABTS^{\bullet+}$, FRAP, and RP assays, closely followed by anthocyanin extracts from the bran and whole grain. Notably, the purified anthocyanin extract displayed high $DPPH^{\bullet}$ activity, which decreased at concentrations above 100 µg/ml. These findings are consistent with previous studies (Bellido & Beta, 2009; Kim et al., 2007; Zhang et al., 2020).

Jin et al. (2022) analyzed the composition of polyphenols and anthocyanins and their antioxidant activity in coloured barley varieties. The findings revealed that purple barley had the highest amount of anthocyanins, significantly surpassing the blue variety. Interestingly, purple and blue barley had similar phenolic compounds, flavonoids, and anthocyanins. However, antioxidant activity assessments revealed a different trend. In terms of iron ion reduction capacity and $ABTS^{\bullet+}$, purple barley displayed significantly higher activity than blue and yellow varieties, and its activity was comparable to that of black barley, consistent with earlier work (Zhang et al., 2020). Conversely, the $DPPH^{\bullet}$ results contradicted the findings of Kim et al. (2007).

Table 1.3. Antioxidant Activity, Detection, and Extraction Methods of Anthocyanins

State of raw barley	Extraction Method	Detection Method	Antioxidant activity assay	Findings	Reference
Hulled and unhulled purple barley	80% methanol containing 0.1% HCl	HPLC-UV-vis detector	DPPH•	There is a correlation between phenols, anthocyanins and antioxidant activity.	Kim et al. (2007)
Purple waxy hull-less barley cv.	3% trifluoroacetic acid (TFA)-50% acetonitrile, 20% ethanol	HPLC- Diode array detector (DAD), Electron spray ionization time-of-flight MS (ESI-TOF MS), High resolution, Fourier transform ion-cyclotron resonance (FT-ICR), NMR	NR	Malonylated cyanidin derivatives are affected by harvest time, drying temperature, and pearling yield.	Kohyama, Ono, & Yanagisawa, (2008)
Purple barley with and without the outer kernel layers	85% methanol containing 15% 1 N HCl	HPLC-photodiode array detector	ORAC	12 peaks/compounds detected but not identified in the hull and 3 in the bran fraction	Bellido & Beta 2009
Hulless purple barley	Acetone, chloroform, and 0.01% HCl	LC-MS	DPPH•, Superoxide radical scavenging capacity assay	One anthocyanin has an alpha bond, and the second has a beta bond. Four unknown compounds, cyanidin and two peonidin derivatives (~40% of total anthocyanins).	Lee, Han, Kim, Baek, & Baik, (2013)
Hulless purple barley	85% methanol containing 15% 1 N HCl	UHPLC-MS	NR	Effects of DNA modifications on anthocyanins content	Zhang, Jiang, Wei, & Liu, (2017)

Table 1.3 cont.

State of raw barley	Extraction Method	Detection Method	Antioxidant activity assay	Findings	Reference
Purple barley without 30% from the outer kernel layers	Different extraction methods were examined	LC-MS	$ABTS^{\bullet+}$, FRAP, RP, $DPPH^{\bullet}$	The extracts contain both acylated and non-acylated, which have the potential for anti-biofilm activity.	Zhang et al. (2020)
Pearled	90% ethanol contains 1% HCl	UPLC-MS QTOF	$ABTS^{\bullet+}$, $DPPH^{\bullet}$, RP, FRAP	Positive effect on reducing hypoxic damage in PC12 cells	Zhang et al. (2021)
NR	0.1% HCl, ultrasonication	HPLC-MS/MS	FRAP, $DPPH^{\bullet}$, $ABTS^{\bullet+}$	Purple barley has the highest antioxidant activity, opposite to anthocyanin content.	Dang, Zhang, Zhang, Yang, & Xu (2022)
NR	Methanol	UHPLC DAD $ABTS^{\bullet+}$ with Accurate-Mass LC/MS Q-TOF	FRAP, $DPPH^{\bullet}$, $ABTS^{\bullet+}$	Analyze the bioaccessibility of anthocyanins	Ed Nignpense, Latif, Francis, Blanchard, & Santhakumar, (2022)
NR	0.1% HCl, ultrasonication	LC-MS/MS	$DPPH^{\bullet}$, FRAP, $ABTS^{\bullet+}$	The high content of anthocyanins is not linked to high antioxidant activity.	Jin, Dang, Zhang, Zheng, & Yang (2022)

NR- Not Reported

1.2.7.4 Cell Culture

A less conventional approach to evaluating the antioxidant activity of barley, maize, and rice involves cell cultures (Gaxiola-Cuevas et al., 2017; Zhu et al., 2015). Two primary assays assess compound bioavailability and antioxidant activity outside of a living organism (ex vivo) (Oduro-Obeng et al., 2024). One method utilizes Caco-2 cells, a type of human epithelial cell, which has been shown to closely mimic human absorption of the compounds after oral intake (Lea, 2015). Caco-2 cells are gaining popularity in scientific research. Another approach is the Cellular Antioxidant Assay (CAA), which evaluates antioxidant activity ex vivo and provides a more direct comparison to the antioxidant processes that occur in the human body. Various cell types can be used in the CAA, and the results can vary depending on the treatment applied, as demonstrated by McCarthy et al. (2013).

Table 1.4 summarizes selected studies on the antioxidant activity of grains using different cell types. Each cell type has unique characteristics. For example, Jurkat T cells are human leukemic T cell lines, while U937 cells are monocytic lymphoma cell lines (McCarthy et al., 2013). HepG2 cells are human liver cancer cells (Zhu et al., 2015), and red blood cells are commonly found in the human body (Arouna, Gabriele, & Pucci, 2020).

Evaluating cellular antioxidant activity involves multiple reagents and can be time-consuming, as the reagents must remain active after being stored at low temperatures. As previously mentioned, phenolic compounds are generally unstable when exposed to heat. Nevertheless, McCarthy et al. (2013) discovered that roasting the barley before beer production did not alter its CAA. The researchers attributed this finding to the possible formation of strong bonds between phenolics and other compounds, which may have protected the phenolics from degradation during the roasting process.

A study comparing yellow, black, and blue barley varieties found that the blue and black varieties had significantly higher levels of TPC, TFC, and TAC compared to the yellow varieties (Zhu et al., 2015). However, the yellow varieties surprisingly showed superior flavonoid CAA quality. This measure assesses CAA values in relation to their phenolic or flavonoid content, indicating that the yellow varieties utilized flavonoids more efficiently for antioxidant activity compared to the pigmented varieties. In contrast, no significant differences were observed among the varieties in phenolic CAA quality (Zhu et al., 2015).

Gaxiola-Cuevas et al. (2017) found that adding lime ($Ca(OH)_2$) during tortilla production significantly improved the retention of TPC in blue maize tortillas compared to white maize tortillas. In contrast to the findings of Zhu et al. (2015), the pigmented maize showed the highest CAA, although no differences were observed in the ABTS antioxidant activity within the same treatment.

In a separate study, Cui et al. (2020) discovered that enriched buckwheat hull flavonoid (EBHF) extracts exhibited the highest $OH^{\bullet}$ radical scavenging activity compared to the purer buckwheat hull flavonoid extracts (HBHF). However, in the DPPH assay, the trend was reversed. Interestingly, both extracts demonstrated similar and higher antioxidant activity than pure extracted flavonoids when exposed to high glucose and H_2O_2 stress (Cui et al., 2020).

As mentioned earlier, there is no direct correlation between TPC and in vitro antioxidant activity. Arouna, Gabriele, & Pucci (2020) supported this claim by finding no significant differences in TPC between germinated and non-germinated sorghum flour. However, they did observe significant differences in antioxidant activity using the DPPH and FRAP assays following treatment. Cui et al. (2020) found that at certain concentrations (0.1 mg/ml and 1 mg/ml), two types of buckwheat extracts (EBHF and HBHF) showed similar cell viability. In contrast, Arouna,

Gabriele, & Pucci (2020) reported that lower concentrations (0.1 mg/ml) of germinated and non-germinated sorghum flour extracts show significant differences. More recent research found a strong correlation between the results of ABTS, DPPH, and FRAP assays and CAA when analyzing five pigmented sorghum varieties (Xiong et al., 2021). Unlike Zhu et al. (2015), Xiong et al. (2021) found that the pigmented sorghum had higher CAA than the white variety. The phenolic compounds in the bran were found to contribute most significantly to the CAA. However, when assessing the quality of cellular antioxidant activity, the white variety showed comparable or even higher CAA than the pigmented sorghum varieties in some cases (Xiong et al., 2021).

In conclusion, the relationship between TPC, anthocyanins, pigmented grains, and CAA remains unclear due to conflicting evidence. The interactions among the compounds in the grain lead to variations in activity and inconsistent results. Additionally, β-glucans may significantly impact antioxidant assays; however, they undergo fermentation in the body before being absorbed. As previously discussed, these interactions can be detected using FT-IR techniques.

Table 1.4. Selected Cellular Antioxidant Methods

Sample	Bioactive compound	Cell type	Findings	Reference
Brewers Spent Grain	Phenols	Jurkat T, U937	Roasting barley to 200°C before brewing did not alter the antioxidant activity in the cells.	McCarthy et al. (2013)
Barley	Phenols and Flavonoids	HepG2	Flavonoids had higher CAA in all the varieties.	Zhu et al. (2015)
Maize Tortilla	Phenols	HepG2	In all the treatments, tortillas from blue maize had significantly higher antioxidant activity than white maize.	Gaxiola-Cuevas et al. (2017)
Buckwheat Hulls	Flavonoids	HepG2	The treatments caused a decrease in cell viability at 200µg/ml.	Cui et al. (2020)
Sorghum	Phenolics	Red blood cells	Germinated sorghum improved the CAA compared to non-germinated until 100µg/ml extract.	Arouna, Gabriele, & Pucci, (2020)
Sorghum	Phenolics	HepG2	Black and Brown have the highest CAA, and the free phenolics in the bran of these varieties have almost three times higher CAA than the bound form.	Xiong et al. (2021)

1.2.8. Physical and Colour Parameters

Understanding how colour and texture affect food products is essential. The colour of grains and pulses is typically attributed to anthocyanins or polyphenols, whereas vegetables like tomatoes derive their colour from lycopene. During food transportation, food products often undergo freeze-thaw cycles, making it important to study their impact on colour and texture. Wang et al. (2022) investigated this effect on red bean steamed buns and observed only minor changes in colour parameters (L*, a*, and b*) across multiple cycles. However, after the final cycle, the buns became significantly harder.

In contrast, Barani, Zhang, Wang, & Devahastin (2020) examined how different organic acids and sucrose affect the anthocyanins in red roses. They found that the yellow-blue colour

parameter (b*) parameter remained relatively unchanged across all treatments, regardless of concentrations. However, the brightness of the colour was significantly affected. A similar pattern was observed by Seo, Moon, & Kweon (2021) when they added purple wheat bran to bread. The yellow-blue colour parameter (b*) remained steady, regardless of the bran concentration. On the other hand, brightness altered significantly with increasing concentrations despite higher TAC and TPC. Notably, the study revealed a clear correlation between the concentration of pigmented bran and antioxidant activity, as measured by DPPH and ABTS methods (Seo, Moon, & Kweon, 2021).

Malting sorghum significantly impacts TAC, TPC, and various colour parameters across different varieties. This suggests that malting is an effective food processing method impacting multiple attributes (Khoddami, Mohammadrezaei, & Roberts, 2017). Adding black bean extract (BBE) to tortillas primarily changed the redness of the tortillas without altering their physical properties (Chávez-Santoscoy, Gutiérrez-Uribe, Serna-Saldivar, & Perez-Carrillo, 2016). Interestingly, higher concentrations of BBE significantly decreased the TAC in tortillas and gluten-free cookies. A later report by Pérez-Alva, Baigts-Allende, Ramírez-Rodrigues, & Ramírez-Rodrigues (2022) found that the relationship between the concentration of pigmented maize in tortillas mainly depends on the specific variety. Nevertheless, as previously shown, concentration plays a significant role in determining TPC and physical parameters.

CHAPTER 2: **PHYSICAL PROPERTIES of PIGMENTED BARLEY TORTILLAS**

2.1 ABSTRACT

Food processing significantly affects the physical properties of products, particularly those containing β-glucans. Changes in β-glucan content, source, particle size and pH levels can alter texture and physical characteristics of the food product. In thin products like tortillas, even slight variations in thickness can notably impact the texture, highlighting the importance of precise processing control. This study assessed the physical properties (colour, size, thickness and texture) of five pigmented barley tortillas prepared under varying pH conditions (pH 1, pH 3, pH 5, and pH 7). CDC Rattan tortillas showed a notable decrease in thickness (3.3 ± 0.3 mm) at pH 1 compared to pH 7 (3.6 ± 0.3 mm), highlighting the significant impact of pH treatment ($p<0.05$). Additionally, tortillas produced at pH 5 were consistently harder and less extensible across all the genotypes. Overall, pH 5 resulted in increased hardness of tortillas across all genotypes, except for the purple ones, which exhibited larger diameters.

Keywords: Barley, Tortillas, pH treatment, Texture, Size.

2.2 INTRODUCTION

Barley is a nutrient-rich cereal, high in dietary fibre, vitamins, minerals, and phytochemicals, offering significant potential to improve human health.

The texture and physical properties of tortillas and baked goods are influenced by factors such as pH, dietary fibre, temperature, cooking duration, and presence of metals. Previous studies reported that dietary fibre influences the texture and colour of tortillas and bread (Ebringerová & Hromádková, 1999; Izydorczyk & Dexter, 2004; Rosicka-Kaczmarek et al., 2016). β-glucan impacts the bread properties, depending on the source and size (Lazaridou & Biliaderis, 2007). Adding yam flour to tortillas improves their dietary fibre content. However, this addition may affect their texture, which is observed as an increase in hardness and a reduction in extensibility (Asiyanbi-Hammed & Simsek, 2020). Conversely, adding black bean extract may have minimal impact on the texture of the tortilla (Chávez-Santoscoy et al., 2016). Previous studies have shown that commercial tortillas tend to have larger diameters, but lower maximum force (indicating softer texture) compared to tortillas made with finer flour particle sizes. Additionally, the redness of tortillas has been linked to their phenolic content (Prasopsunwattana et al., 2009; Asiyanbi-Hammed & Simsek, 2020). Among the colour and texture, the consumer likeness is an important factor in tortillas (Montemayor-Mora et al., 2018).

The study examines the impact of pH on the physical properties of tortillas made from pigmented barley using varying pH levels (pH 1, 3, 5, and 7) in their preparation.

2.3. MATERIALS AND METHODS

2.3.1 Raw Materials

Five barley genotypes (CDC Rattan, CDC McGwire, Roseland, HB21147, and HB21148) were generously provided by Dr. Ana Badea from the Agriculture and Agri-Food Research and

Development Centre in Brandon, Manitoba, Canada (Figure 2.1). The barley characteristics are presented in Table 2.1. HB21148 is not a registered variety. Therefore, I used the term genotype instead of variety for simplicity purposes. All the barley genotypes were developed and grown in Canada and are adapted to the Canadian weather.

Each genotype was milled using a Retsch ZM 200 grinder (Retsch GmbH, Haan, Germany)with a 0.5 mm sieve at 10,000 rpm. The barley flour was stored at 4°C in sealed plastic

Figure 2.1. Barley samples. A- CDC Rattan, B- CDC McGwire, C- Roseland, D- HB21147, E- HB21148

bags until further use.

2.3.2 Tortilla Production

Tortillas were produced following the method described by Méndez et al. (2013) with some modifications. Acidified water at pH 1, 3, 5, and 7 were prepared using 1M HCl. To 100 g of barley flour, 87.4 ml of acidified water was added gradually, mixed, and manually kneaded. Dough balls (15 g) were flattened and cooked in the DoughPro© DP 2000 (Proluxe, Perris, CA, USA) with the upper platen preheated to 121.1°C and the lower platen at room temperature. The cooking

process involved 10 seconds on the first side, 25 seconds on the second side in a preheated pan, and an additional 10 seconds after turning. The tortillas were then cooled to room temperature before undergoing freeze-drying. The freeze-dried samples were subsequently milled

using Proctor Silex® Fresh Grind™ (model 80301C, Hamilton Beach Brands, Glen Allen, VA, USA).

Table 2.1. Characteristics of the Barley Genotypes

Genotype	Ash (%)	Crude protein (%)	β-Glucan	Colour
CDC McGwire	1.74 ± 0.01C	12.12 ± 0.41B	5.0% (Normal)	Yellow
CDC Rattan	1.84 ± 0.01B	12.08 ± 0.39B	7.5% (High)	Yellow
Roseland	1.78 ± 0.00C	12.12 ± 0.57B	4.5-5% (Normal)	Yellow
HB21147	1.94 ± 0.03A	14.42 ± 0.06A	7.1% (High)	Purple
HB21148	1.85 ± 0.00B	15.82 ± 0.23A	5.9% (Normal)	Purple

Different uppercase letters represent significant differences among genotypes.

2.3.3 Physical Parameters

2.3.3.1 Colour Analysis

The colours of the different tortilla samples were measured using Lovibond LC 100 colorimeter (The Tintometer, Amesbury, UK). The instrument analyzed the samples in terms of three-color parameters: L* (lightness): ranging from 0 (dark) to 100 (light), a* (+) red- (-)green axis, and b* (+) yellow- (-) blue axis.

2.3.3.2 Determination of the Texture and Size of the Tortilla Samples

Tortilla texture was evaluated according to the method of Gaspare et al. (2024). Briefly, each tortilla was placed on the heavy-duty platform and analyzed using TA.TXT.Plus texture

analyzer (Stable Microsystem, Surrey, UK). The texture analyzer's built-in tortilla-pastry burst rig program measured the maximum tension force and extensibility, with greater distance at break indicating higher extensibility, as defined by the software.

Tortilla diameter was measured using Traceable™ Digital Callipers, Fisherbrand™, with an accuracy level of 0.01mm. Two measurements were taken for each tortilla, one at the initial position and another after rotating 90° (Prasopsunwattana et al., 2009). An average was calculated from the two readings. The thickness was determined by measuring four random points and calculating the average. Four tortillas were randomly selected for these measurements.

2.3.4 Statistical Analysis

The statistical analysis was performed using the R programming language (R Core Team, 2022). Each dataset was assessed for normality and homogeneity. When assumptions were met, ANOVA was used. For non-normal data, the Kruskal-Wallis and Wilcoxon's test (rstatix package) replaced ANOVA (Bauer, 1972; Irland, 2010). To control for type 1 error inflation in Wilcoxon's test, the Benjamini & Hochberg correction was employed (Benjamini & Hochberg, 1995). A significance level of 5% was used.

2.4 RESULTS AND DISCUSSION

2.4.1 Tortilla Physical Parameters

To minimize the impact of extraneous compounds, tortillas were made using only water and barley flour, as previously described (Enaru et al., 2021; Gamage & Choo, 2023; Hou et al., 2013). The prepared tortillas are shown in Figure 2.2. During production, Roseland produced a more extensible and uniform dough after kneading, resulting in tortillas with a superior appearance compared to other barley genotypes. A detailed discussion of tortilla properties is presented below.

2.4.2 Colour Parameters

The colour parameters (L*, a*, b*) of the tortillas were expressed using the CIE L*a*b* scale (Table 2.2) for several reasons. Firstly, the natural contrast between genotypes makes differences easier to detect. Secondly, ΔE is not suitable due to the assessment of purple using the +a* Hunter scale. Lastly, the +b* scale (blue to yellow), reveals greater sensitivity to yellowness variations in three genotypes. Notably, CDC Rattan and CDC McGwire tortillas had similar brightness (L* parameter) across all pH levels, while the b* parameter was similar in all yellow genotypes under different pH treatments. However, dietary fibre concentrations significantly affected the L* and a* parameters compared to the control, with minimal impact on the b* parameter (Hernández-Reyes et al., 2019). Despite significant differences, CDC McGwire and CDC Rattan tortillas showed close a* values at the same pH level consistent with findings by Vazquez-Villegas et al. (2021). Despite significant differences in the a* and b* parameters after addition of protein and dietary fibres to tortillas, human assessment did not find these changes in the colour, flavour and overall tortilla quality (Montemayor-Mora et al., 2018).

2.4.3 Tortilla Sizes

The diameter and thickness of the tortillas were assessed using digital callipers, with results reported in Table 2.2.

Roseland tortillas were significantly larger in diameter and thinner than CDC Rattan tortillas across all the pH levels. HB21148 tortillas had a significantly larger diameter (67.9 ± 1.0 mm) than CDC Rattan (65.4 ± 1.0 mm) at most pH levels, except at pH 1. No differences were observed in diameter and thickness among Roseland, CDC McGwire, and HB21147 tortillas at the same pH level. Except for CDC Rattan, the treatment did not affect the thickness of the tortillas. CDC McGwire had significantly smaller tortillas at pH 5 (66.5±0.9 mm). CDC Rattan tortillas at

pH 7 (64.2±1.4 mm) were significantly smaller than at pH 1 (67.9 ± 1.0 mm). No significant differences in tortilla diameter and thickness among the HB21148, HB21147, and Roseland genotypes were observed. The original tortillas in Mexico are 2 mm thick and 15-33 cm diameter (Montemayor-Mora et al., 2018), which are thinner and bigger than the produced for this research. Production of these tortillas needed 100g per treatment per genotype with 15g per tortillas; the bigger the tortilla, we would need significantly higher amounts of the barley for each trail.

These findings are consistent with previous research on the role of acids and pH levels in baked products. Additionally, Hernández-Reyes et al. (2019) found that adding dietary fibers increased tortilla diameter but not thickness. According to Cauvain & Young (2006), ascorbic acid significantly impacts bread and fermented products, while other baking acids may have different effects. More recent work highlights the importance of acids in achieving softer textures and larger volumes in fermented products, suggesting that both acid type and pH level play a role (Asamoah et al., 2023). Their findings suggested that the size is acid-dependent, but the pH level also has a role. Additionally, Hernández-Reyes et al. (2019) found that adding dietary fibres in soybean bagasse did not change the tortilla thickness but significantly increased the tortilla diameter.

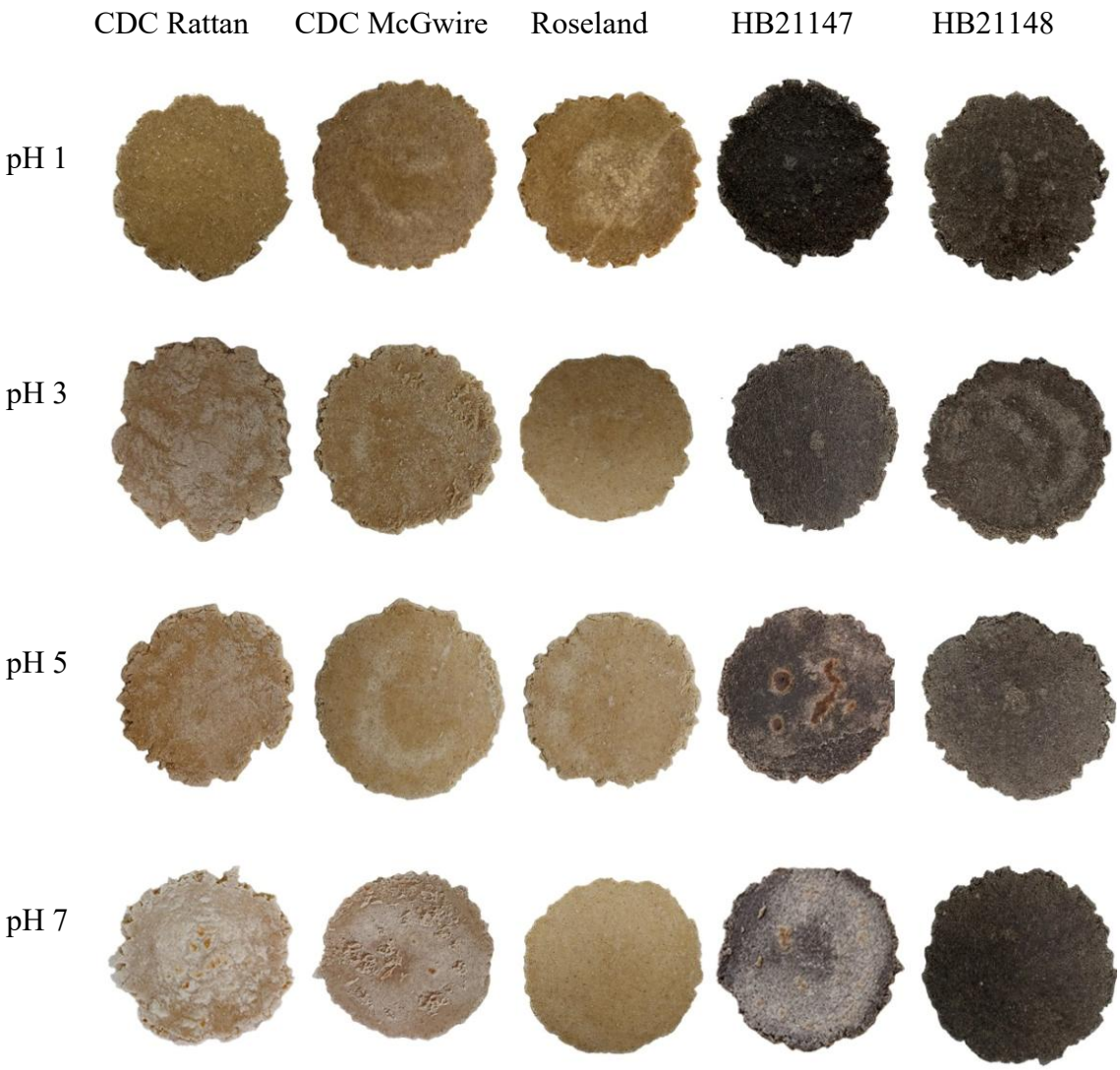

Figure 2.2. Barley tortillas prepared under different pH conditions.

2.4.4 Tortilla Texture

Tortilla texture was evaluated by measuring extensibility and toughness, as shown in Table 2.3. Extensibility refers to the tortilla's ability to stretch under pressure (Gasparre et al., 2024), while toughness or hardness refers to the force required to fracture the tortilla (Argüello-García et al., 2017). As rollability is also a common test texture test for tortillas (Mao & Flores, 2001; Menchaca-Armenta et al., 2020; Montemayor-Mora et al., 2018), but tortilla texture can be measured by extensibility, as was reported before using different names (Osorio-Díaz et al., 2010;

Prasopsunwattana et al., 2009). Hardness was the measurement reported in that all the cited papers above, with unit variations.

Notably, HB21148 tortillas showed consistent toughness across all pH levels, with the highest value at pH 1 (640 ± 237g) and slightly lower values at pH 3, 5 and 7, suggesting minimal impact from pH treatment., Tortillas made from most genotypes exhibited significantly higher toughness values at pH 5, including CDC Rattan (700 ± 119g), CDC McGwire (590 ± 115g), Roseland (505 ± 36g), and HB21147 (657±131g). In contrast, HB21148 tortillas did not exhibit this trend. Additionally, some genotypes showed lower toughness values at specific pH levels, such as Roseland at pH 7 (386 ± 55 g) and CDC McGwire at both pH 3 and 7 (376 ± 71 g and 396 ± 41 g, respectively). These findings suggest a pH-dependent response with pH 5 notably increasing toughness in tortillas from all genotypes except HB21148.

All the genotypes exhibited similar extensibility in tortillas at pH 1 and pH 7. At pH 5, only CDC Rattan (6.6±0.7 mm) and Roseland (6.0±0.7 mm) had a significantly different extensibility values. The treatment had a similar effect on extensibility in all the genotypes in pH 1 and pH 3, but pH 5 and pH 7 resulted in significantly lower extensibility compared to pH 1 and pH 3. HB21148 and CDC McGwire had the lowest extensibility in pH 5 (6.3±0.8mm and 6.3±0.9mm, respectively). Notably, lower toughness was generally associated with higher extensibility, except for HB21148. These findings are consistent with previous research, such as Vázquez-Villegas et al. (2021), which found similar patterns regardless of the fibre content in the tortillas over one week of storage. Additionally, Pérez-Alva et al. (2022) suggested that colour may influence texture; red wheat produced considerably harder tortillas than blue wheat across all levels of seaweed (%) addition. Barley is known to have high amounts of starch. Cooperating starch from red and white wheat into tortillas, did not alter the stretchability (Wang & Flores, 1999). The

protein content plays a critical role in bake products as they denatured during the baking (Pagani et al., 2013). As mentioned in Table 2.1, the protein content of the purple genotypes are significantly higher the yellow once. However, no constant results were found among the genotypes. Notably, no significant differences were found between genotypes in pH 1 and pH 7 at the extensibility parameter (Table 2.1). On the other hand, it is necessary to remember that tortillas are being produced by three methods hot-press, die-cut or handstretch as explained by Barros et al. (2010). Therefore, the reported results were made after hand-made tortillas, so changing the procedure may impact the results. This section demonstrated that it is possible to produce tortillas with high β-glucan concentration when they are naturally presented in the barley grain without significantly affecting the texture, which may overcome the reported challenges (Alviola, Jondiko & Awika, 2010).

Table 2.2. Colour and Size Parameters of the Tortilla Samples

Genotype	Treatment	L	a §	B	Diameter (mm)	Thickness (mm)
HB21148	pH 1	62.6 ± 0.4bD	3.4 ± 0.1aC	9.9 ± 0.2aB	65.4 ± 1.0 bAB	3.1 ± 0.1aABCD
	pH 3	64.1 ± 0.8aB	2.7 ± 0.1bB	9.6 ± 0.0bB	70.8 ± 2.0aABD	3.0 ± 0.2aABCE
	pH 5	63.3 ± 0.4aB	2.7 ± 0.0bC	9.7 ± 0.1abB	70.7 ± 1.2aABD	2.8 ± 0.1bC
	pH 7	63.6 ± 0.3aC	2.7 ± 0.1bC	9.8 ± 0.1abB	67.2 ± 0.8bABD	3.1 ± 0.0aABCE
HB21147	pH 1	65.7 ± 0.3aC	2.6 ± 0.1aE	8.7 ± 0.0bC	67.8 ± 2.0aAB	3.2 ± 0.1aABCD
	pH 3	65.6 ±0.4aB	2.4 ± 0.1aB	9.0 ± 0.1abC	70.2 ± 1.0aABC	3.3 ± 0.2aACDE
	pH 5	64.7 ± 1.2aB	2.6 ± 0.1aBC	9.4 ± 0.3aB	67.7 ± 1.7aABD	3.0 ± 0.2aCD
	pH 7	66.5 ± 0.8aD	2.4 ± 0.1aB	9.4 ± 0.3aB	67.8 ± 0.5cABD	3.1 ± 0.1 aABCE
Roseland	pH 1	79.6 ± 0.5aA	3.4 ± 0.1aD	14.2 ± 0.1cA	69.4 ± 0.7bBC	2.7 ± 0.0bBE
	pH 3	79.4 ± 0.5aA	2.7 ± 0.1bBC	14.2 ± 0.1bcA	72.9 ± 1.0aBD	2.8 ± 0.2bBE
	pH 5	77.4 ± 0.5bA	2.9 ± 0.1bC	14.7 ± 0.1abcA	69.4 ± 1.4bBD	2.8 ± 0.1bBC
	pH 7	77.8 ± 0.9abA	2.8 ± 0.2bAC	14.9 ± 0.5aA	70.4 ± 1.4BD	3.1 ± 0.0aE
CDC McGwier	pH 1	77.4 ± 0.8B	3.2 ± 0.0abcC	13.7 ±0.2bA	70.5 ± 1.5aC	2.6 ± 0.1cE
	pH 3	77.1 ± 0.9A	2.6 ± 0.2aC	13.9 ± 0.1abA	72.8 ± 1.0abD	2.7 ± 0.1cB
	pH 5	77.9 ± 0.8A	2.8 ± 0.1abBC	14.3 ± 0.3aA	66.5 ± 0.9bDE	3.2 ± 0.1aAC
	pH 7	77.2 ± 0.8B	2.8 ± 0.1cB	14.6 ± 0.2	69.8 ± 1.7abD	2.9 ± 0.0bE
CDC Rattan	pH 1	77.5 ± 0.5abB	3.7 ± 0.1aA	14.2 ± 0.2bcA	67.9 ± 1.0aA	3.3 ± 0.3bD
	pH 3	77.7 ± 0.5aA	3.2 ± 0.1bA	14.2 ± 0.2bdA	68.7 ± 1.2abC	3.5 ± 0.4abD
	pH 5	76.5 ± 0.9bA	3.3 ± 0.1abcA	15.3 ± 0.2a	62.5 ± 1.8abCE	3.6 ± 0.3abA
	pH 7	77.1 ± 0.5abA	3.2 ± 0.1cA	14.7 ± 0.1cdA	64.2 ± 1.4bC	3.6 ± 0.3aD

Different lowercase letters represent significant differences within the genotype. Different uppercase letters represent significant differences between genotypes at the same pH level. n=4 § the analysis was made by Wilcoxson`s test.

Table 2.3. The Texture of the Tortilla Samples

Genotype	Treatment	Toughness § (g)	Extensibility§ (mm)
HB21148	pH 1	640 ± 23 aAB	9.9 ± 1.7 aA
	pH 3	544 ± 77aA	9.0 ± 0.4 aABC
	pH 5	534 ± 70 aC	6.3 ± 0.8 bAB
	pH 7	564 ±65 aB	6.4 ± 0.4 bA
HB21147	pH 1	513 ± 69 bB	8.9 ± 0.7 aA
	pH 3	526 ± 62 bAB	8.9 ± 0.6 aAC
	pH 5	657 ±131 aA	6.2 ± 0.7 bA
	pH 7	512 ± 63 bB	6.0 ± 0.8 bA
Roseland	pH 1	470 ± 89 bAB	9.2 ± 0.3 aA
	pH 3	421 ±77 bcCD	8.8 ± 0.4 aABC
	pH 5	505 ± 36 aB	6.0 ± 0.7 bB
	pH 7	386 ± 55 cC	7.2 ± 0.8 bA
CDC McGwire	pH 1	473 ± 119 abAB	9.5 ± 0.4 aA
	pH 3	376 ± 71 aD	8.7 ± 0.9 aAC
	pH 5	590 ± 115 bAC	6.3 ± 0.9 bAB
	pH 7	396 ± 41 aC	6.5 ± 1.2 bA
CDC Rattan	pH 1	687 ± 98 aA	8.8 ± 0.5 aA
	pH 3	473 ± 86 bBC	8.8 ± 0.6 aB
	pH 5	700 ± 119 aAB	6.6 ± 0.7 bA
	pH 7	686 ± 54 aA	6.7 ± 0.7 bA

Different lowercase letters represent significant differences within the genotype. Different uppercase letters represent significant differences between genotypes at the same pH level. n=10, § the analysis was made by the Wilcoxson's test.

2.4 CONCLUSIONS

The pH level had a significant effect on the physical characteristics of the tortillas. Among the different genotypes, notably HB21148 tortillas were 0.8 mm thinner than CDC Ratan at pH 5. CDC McGwire and CDC Rattan also showed smaller diameters at pH 5, measuring 66.5 ± 0.9 mm and 62.5 ± 1.8 mm, respectively. Both pH 5 and pH 7 resulted in similar extensibility across all barley tortilla genotypes. These findings suggest that the texture and physical properties of the tortillas are influenced by the pH level applied during their preparation. Given these effects, further assessment of the antioxidant compounds and antioxidant activity in tortillas is warranted.

CHAPTER 3: **PIGMENTED BARLEY TORTILLAS: ANTHOCYANINS, POLYPHENOLS CONTENT AND THEIR ANTIOXIDANT ACTIVITY *IN VITRO* AND *EX VIVO***

3.1 ABSTRACT

This chapter investigated the anthocyanin and polyphenolic content, as well as the *in vitro* and *ex vivo* antioxidant activity of pigmented barley tortillas. CDC McGwire tortillas at pH 5 had significantly higher total phenolic content (10.9±1.8mg gallic acid equivalent/g DW) compared to other pH levels within the genotype. In contrast, the pH treatment significantly decreased the total anthocyanin content (TAC) in all the genotypes, with CDC McGwire tortillas at pH 1 having the lowest content (16.7±10.2 Cya-3-O eq µg/Kg DW). The purple genotypes HB21147 and HB21148 tortillas consistently showed higher antioxidant activity (AA) using the DPPH method across all pH treatments. In contrast, CDC Rattan and CDC McGwire tortillas demonstrated lower AA compared to Roseland and purple genotypes in FRAP and ABTS assays. These findings indicate that pH levels have a significant impact on both physical and AA properties of tortillas, affecting more than just anthocyanins content. The cell AA assay suggested that naturally occurring anthocyanins may have distinct antioxidant mechanisms compared to those of CDC Rattan, particularly at concentrations higher than 100 µg/ml. The study found that barley tortillas produced under pH 3 had higher TAC and AA. Our analysis suggests that low pH levels positively impact barley tortillas by enhancing their phenolic content and AA. Therefore, such food products potentially make them a healthier option.

Keywords: Antioxidant activity, cell culture, anthocyanin stability

3.2 INTRODUCTION

Phytochemicals, especially anthocyanins, may benefit human health by reducing the risk of cardiovascular diseases and cancer (Idehen, Tang, and Sang, 2017; Martínez et al., 2018). These compounds, which contribute to the colour of grains, exhibit antihypertension, anti-diabetic, anti-inflammatory, and anti-obesity effects (Delgado-Povedano et al., 2021; Ed Nignpense et al., 2022).

In barley, the primary anthocyanins include cyanidin-3-O-glucoside, pelargonidin-3-O-glucoside, and peonidin-3-O-glucoside (Dang et al., 2022; Zhang et al., 2017). Their stability is pH-dependent, with pH 1 helping to preserve the colour, whereas pH 4.5 and pH 7 result in colour loss (Ed Nignpense et al., 2022; Chen et al., 2022).

Factors such as pH, dietary fibre, temperature, cooking duration, and metals influence the texture and physical parameters of tortillas and baked products. Previous studies have shown that dietary fibre, in particular, can impact the texture and colour of tortillas and bread (Ebringerová & Hromádková, 1999; Izydorczyk & Dexter, 2004; Rosicka-Kaczmarek et al., 2016). β-glucan affects bread properties, with its impact varying depending on the source and size (Andrzej et al., 2020; Lazaridou & Biliaderis, 2007). Additionally, incorporating yam flour into tortillas can increase their dietary fibre content, although it may also increase hardness and reduce extensibility (Asiyanbi-Hammed & Simsek, 2020). Conversely, adding black bean extract does not significantly alter the texture of the tortilla (Chávez-Santoscoy et al., 2016). Previous studies have shown that commercial tortillas tend to have larger diameters but require less force, compared to tortillas made with flour of smaller particle sizes, (Prasopsunwattana et al., 2009; Asiyanbi-Hammed & Simsek, 2020).

Grains are usually consumed after cooking/processing and rarely in raw form. However, the processing can affect anthocyanin concentration and activity in the food matrix (Seo, Moon,

& Kweon, 2021; Tuersuntuoheti et al., 2021). For instance, after malting pigmented sorghum, the content of anthocyanins can be increased or decreased (Kohyama, Ono, & Yanagisawa, 2008; Méndez Lagunas et al., 2022). Fermentation time also affects the pH, influencing anthocyanin concentration (Francavilla & Joya, 2022). Due to the effects of different processing methods on anthocyanin content in food products, several approaches were suggested to improve anthocyanin stability during processing (Chen et al., 2022). A short cooking duration and optimal pH (1~3) could significantly reduce anthocyanin degradation (Chavez-Santoscoy et al., 2016; Gamel et al., 2023; Hou et al., 2013; Petersson et al., 2010).

Antioxidant activity is typically assessed using in vitro methods, which are valued for their speed, cost-effectiveness, reliability, and reproducibility (Bibi Sadeer et al., 2020). However, these methods do not accurately mimic the human physiological environment, owing to their distinct mechanisms of action (Wolfe & Liu, 2007). There is a growing need for antioxidant testing methods that better mimic physiological conditions. This study focuses on the identification and quantification of anthocyanins and polyphenols in raw materials and tortillas at various pH levels, using both in vitro and ex vivo antioxidant activity assays.

3.3. MATERIALS and METHODS

Sections 2.3.1 and 2.3.2 outline the selected barley genotypes and tortilla production.

3.3.1 Extraction and quantification of total phenolic content (TPC) and total anthocyanin content (TAC)

Raw barley and tortilla samples were extracted following the method described by Jin et al. (2022) with some adjustments. For the procedure, 2 g of sample were extracted in 10 ml of acidified ethanol (1% HCl) using 50 ml centrifuge tubes. The tubes were shaken for one hour (Burrell Scientific Wrist Action™ Model 75 Laboratory Shakers, Thomas Scientific, Swedesboro,

NJ, USA), followed by sonication for 30 minutes (Branson Ultrasonics™ 5510 Bransonic™, Richmond, VA, USA). The supernatant was then transferred to clean 50 ml centrifuge tubes. The process was repeated twice and centrifugation carried at 8000 rpm for 20 min at 25°C. After centrifugation, the supernatant was filtered using Choice© 25 mm, 0.45 µm nylon (Thermo-Scientific, Waltham, MA, USA). The filtered solution was evaporated using a rotary evaporator set at 40°C and 200 rpm. Then, the concentrated samples were kept at -20°C until further analysis.

TPC was determined using the method described by Apea-Bah, Drawbridge, & Beta (2022). A sample extract (18.2 µl), 36.4 µl of 10% (v/v) aqueous Folin-Ciocalteu, and 145.5 µl of 700 mM sodium carbonate were added to a 96-well plate and incubated in the dark for 60 min. After incubation, the plate was read in a microplate reader at 750 nm. The calibration curve was made with different concentrations of gallic acid ranging from 0.025 to 0.15 mg/ml. All the samples were diluted 20 times to fall within the range of the calibration curve. The results were expressed in mg GAE/g DW (milligram of gallic acid equivalent in gram of dry weight). TPC is working based on the SET when the Folin−Ciocalteu is reduced with phenolic compounds in an alkaline medium and the spectrophotometric reading measures the colour change duo to this reduction (Molole et al., 2022). Despite ferulic acid is the common phenolic compound in barley, I used gallic acid since it is more common in other food products and plants and the comparison is more robust.

TAC was determined according to Lee et al. (2005). Briefly, each sample was mixed with pH 1 buffer (potassium chloride, 0.025M) and pH 4.5 (sodium acetate, 0.4M), and the absorbance was read at 520 nm and 700 nm for each buffer after 20 minutes of incubation in the dark. The pH buffers are to change the chemical structures of anthocyanins as cyanidin-3-glucoside has peak absorbance at 520nm. The 700nm reading is to overcome the “cloudiness” and not for the

anthocyanins. The results were expressed as mg Kg^{-1} cyanidin-3-glucoside equivalent (C-3-G). In case the suspect anthocyanin is different then C-3-G, the peak wave length must be adopted to the anthocyanin.

3.3.2 HPLC analysis

To assess the impact of the pH levels on anthocyanins, tortillas enriched with anthocyanin standards were prepared, and the raw flour, dough, and tortillas were analyzed using HPLC as per the method outlined by Bellido & Beta (2009).

The phenolic acid analysis was made using a previous protocol in our lab (Drawbridge, Apea-Bah, Silveira Hornung, & Beta, 2021). Identification and quantification were conducted using standards from Sigma Aldrich, USA (catechin, 3,4 dihydroxy benzaldehyde, gentisic acid, p-hydroxybenzoic acid, 4-hydroxybenzaldehyde, p-coumaric acid, m-coumaric acid, o-coumaric acid, chlorogenic acid, naringin, ellagic acid, trans-cinnamic acid and quercitin dihydrate).

Dr. John Sorenson (Chemistry Department, University of Manitoba) generously provided two reference standards: cyanidin 3-O-glucoside (USP, Rockville, MD, USA) and cyanidin 3-O-galactoside (PhytoLab, Vestenbergsgreuth, Germany). Each standard (2 mg) was added to CDC Rattan flour.

3.3.3 Antioxidant Assays

The antioxidant activity of the different samples was assessed through three assays, as described below.

The FRAP assay was conducted according to Basheer et al. (2021) with some adjustments. The FRAP reagent was prepared by mixing 200 ml of 300 mM buffer acetate (pH 3.6), 20 ml of 10 mM TPTZ solution and 20 ml of 20 mM $FeCl_3$ solution (10:1:1). The mixture was incubated at 37°C for 30 min, and the working solution was used within three hours. For the assay, 30 µl of the

sample was mixed with 1.47ml of working FRAP solution and incubated in the dark for 30 min. The absorbance was read at 593 nm using a spectrophotometer.

TEAC/ABTS and DPPH were made according to the protocol from our lab (Apea-Bah, Drawbridge, and Beta. 2022). The ABTS assay was performed by mixing 16 mM ABTS stock solution with 6 mM potassium persulfate in equal amounts and incubating for 12 hours to create an ABTS working solution. After incubation, the working solution was diluted 60 times with 200 mM PBS to reach an absorbance of 0.8 with a spectrophotometer. To measure antioxidant activity, 10 µl of extract was combined with 190 µl of ABTS working solution in a 96-well plate. The mixture was then incubated in the dark for 60 min, after which absorbance was read at 750nm. The DPPH assay was conducted in a 96-well plate by mixing 195µl of 60µmol/l of DPPH solution with 5µl of sample extract or different concentrations of Trolox standard. The mixture was incubated for 60 min in the dark. After incubation, the reading was taken at 515 nm. All AA assays results were expressed as µg Trolox eq/g DW.

3.3.4 Determination of Barley Antioxidant Activity using Caco-2 Cell Model

3.3.4.1 Sample Preparation

The raw extract was used, as explained in section 3.3.1. Following the rotary evaporator, the samples were completely dried using N_2 gas and stored at -80°C.

3.3.4.2 Cell Culture Conditions

The Caco-2 cell line used in this study was obtained from the American type culture collection (ATCC) (Manassas, VA, USA) and generously provided by Dr. Chengbo Yang (Animal Science, University of Manitoba, Winnipeg, Canada) at passage 8. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotics (penicillin-streptomycin). To ensure optimal growth of the cells, the incubator

was set at 5% CO_2, 90% humidity, and 37°C. The cell culture medium was replaced every three days until the cells reached approximately 90% confluence.

3.3.4.3 Determination of Cell Viability via MTT Assay

The viability of Caco-2 cells was assessed by the MMT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) method according to Zongo et al. (2022) with some modifications. When the cells reached 80 – 90% confluence, they were washed twice with PBS and detached from the culture flask bottom using trypsin-EDTA (0.25% trypsin with 1 mM EDTA).They were then placed in the incubator for 8 min before adding the DMEM containing FBS to neutralize the trypsin and resuspend the Caco-2 cells. For the assay, cells were plated in 96-well plates at a density of 1×10^4 cells per well (100 µl/well) and incubated for 24 h to allow attachment. After 24h, the culture medium was carefully removed, and cells were incubated with different concentrations of barley HB21148 extract (25 – 800 µg/mL) or barley CDC Rattan+ACN extract (25 – 800 µg/mL) for 24h. Thereafter, the treatment solutions were cautiously sucked out, and cells were incubated with 120 µL per well of MTT solution (0.5 mg/mL) for 3 h. Then, the MTT was removed, and 150 µl of HPLC-grade DMSO was added to dissolve the salt. The microplate was allowed to stay in the dark for 10 min at room temperature. Finally, the 96-well plate was shaken for 5 min in a microplate reader, and the absorbance was measured at 490 nm.

The cell viability rate was calculated using the following formula:

$$\textbf{\% Cell viability} = ((\textbf{Abs}_{\textbf{sample}} - \textbf{Abs}_{\textbf{blank}})/(\textbf{Abs}_{\textbf{control}} - \textbf{Abs}_{\textbf{blank}})) \times 100$$

3.3.4.4 Cellular Antioxidant Activity (CAA) assay

The CAA assay was performed following the method described by Wolfe and Liu (2007). The barley extracts were diluted in serum-free DMEM to prepare different concentrations (50, 100, and 200 µg/mL) and catechin (6.25, 12.5, 25, 50, 100, and 200 µM) was also used as standard

for this assay. A toxic compound, 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) was prepared at a concentration of 200 μM. For the procedure, Caco-2 cells were seeded in a black 96-well microplate with clear bottom at a density of 2×10^5 cells per well (100 μl/well) and incubated for 24 h. After removing the culture medium, cells were washed once with 100 μL PBS. The probe, dichlorodihydrofluorescin diacetate (DCFH-CA) (2.5 μM, 50 μl) and 50 μl of catechin standard or barley extract were added into each well and incubated at 5% CO_2, 37°C for 1h. After incubation, the treatments were carefully removed, and the cells were gently washed one time with 100 μl PBS. To initiate the assay, 100 μl of AAPH (200 μM) was added to each well, and the plate was placed in a 37°C microplate reader to begin kinetic fluorescence measurements using excitation at 485 nm and emission at 538 nm. The reading was taken every 5 min for a total of 60 min. The assay was carried out in triplicate for each concentration, and the area under the curve (AUC) were calculated for each sample and standard using the relative fluorescence unit values. The CAA units were determined following the formula and the AUC values:

$$\textbf{CAA Units} = 100 - ((AUC_{Antioxidant}/AUC_{Control}) \times 100)$$

3.3.5 Statistical Analysis

The statistical analysis for cellular antioxidant activity was performed with GraphPad Prism 10.2.0. The results are expressed as mean ± standard deviation (SD). Statistical difference between groups was determined by t-test and considered significant at $\alpha < 0.05$. The other parts were analyzed as described in section 2.3.4.

3.4 RESULTS AND DISCUSSION

3.4.1 Total Phenolic Content (TPC)

The total phenolic content (TPC) of the raw barley and tortilla samples are shown in Figure 3.1. Among the raw barley, HB21148 had the highest TPC at 14±1.2 mg GAE/g DW. At pH 7, the

TPC varied among tortilla samples. HB21147 had the highest TPC at 10.4 ± 1.1 mg GAE/g DW, while CDC Rattan and CDC McGwire had significantly lower TPC values of 2.6 ± 0.8 and 4.4 ± 0.7 mg GAE/g DW, respectively. The TPC of Roseland was 8.1±2.3 mg GAE/g DW. No significant differences in TPC were found between HB21147 and Roseland which showed similar TPC levels across all pH levels. Additionally, HB21148, Roseland, and CDC Rattan had comparable TPC at pH 1, 3, and 5. Our results also showed no significant differences in TPC at pH 1 in all the genotypes. The TPC for HB21147 remained stable across all pH treatments, showing no significant differences. The TPC in CDC McGwire significantly increased to 10.9±1.8 mg GAE/g DW at by pH 5, compared to the other pH levels.

Research findings on the impact of pH on TPC have been mixed. Gamage & Choo (2023) found that pH 3, 4, and 5 did not affect TPC during storage at 4°C. In contrast, Pozo-Insfran et al. (2007) reported that pH 5.2, significantly increased TPC and TAC in blue corn doughs and tortillas compared to white corn. On the other hand, barley colour does not directly show correlation with TPC, as studies have shown some purple varieties have higher phenolic content than yellow

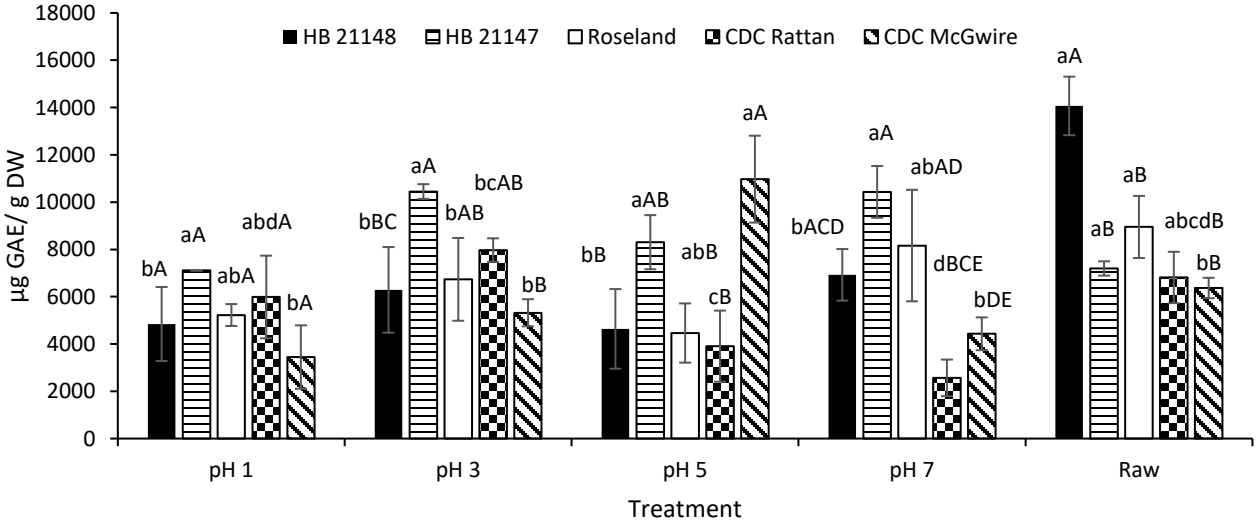

Figure 3.1. Total Phenolic Content of raw barley genotypes and tortillas. Data is presented as mean ± SD; n=3. Different lowercase letters represent significant differences within the genotype. Different uppercase letters represent significant differences between genotypes at the same pH level.

varieties, and some yellow varieties have higher TPC than purple and blue varieties (Dang et al., 2022). A previous study also found that yellow barley varieties had higher TPC than purple barley varieties (Deng et al., 2021). Interestingly, a study by Shin & Lee (2014) reported a significant correlation between β-glucan content and TPC.

3.4.2 Total Anthocyanin Content (TAC)

The total anthocyanin content of the processed tortillas and raw barley genotypes is expressed as cyanidin-3-glucoside (C-3-G) eq in mg/Kg DW (Figure 3.2). HB21148 exhibited significantly higher TAC across most treatments, except at pH 5, with an overall average of 115.8±11.3 mg C-3-G eq/Kg DW. Notably, the highest TAC values for HB21148 and CDC McGwire were found in their raw forms, at 313.9±33.4 and 136.9±16.7 mg C-3-G eq/Kg DW, respectively. Contrary to expectations, no correlation was observed between the pH and TAC. This finding differs from Martínez-Subirà et al. (2020), who reported a four-fold increase in TAC when 0.5% tartaric acid was added to purple barley biscuits. Yu & Beta (2015) observed that the TAC

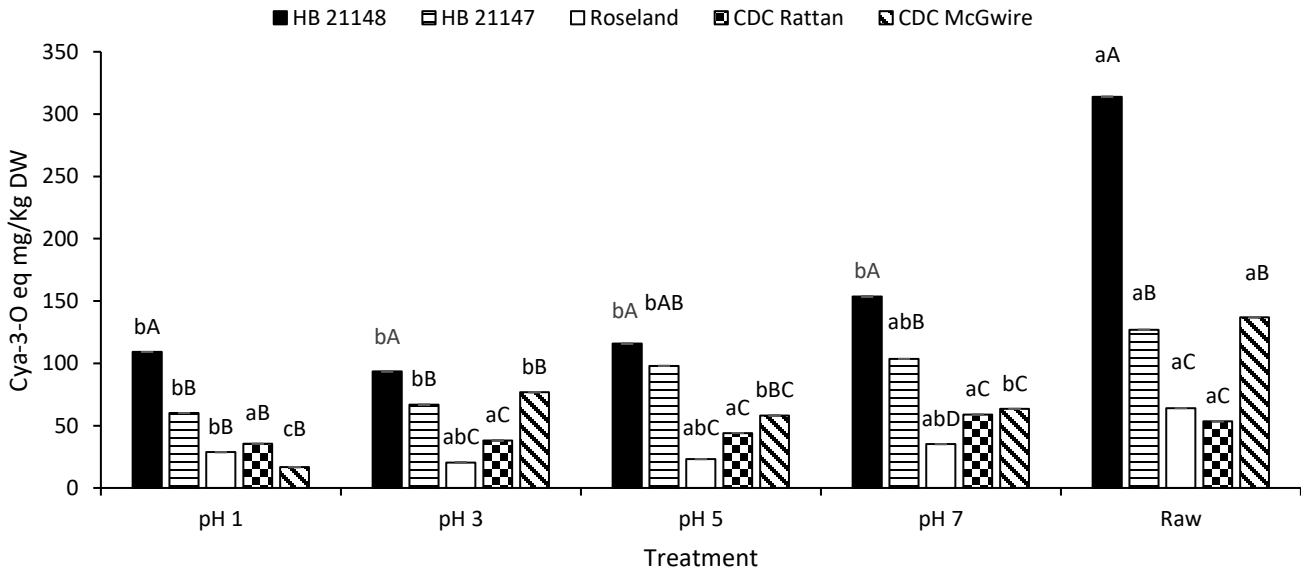

Figure 3.2. Total Anthocyanin Content of raw barley genotypes and tortillas. Data is presented as mean ± SD; n=3. Different lowercase letters represent significant differences within the genotype. Different uppercase letters represent significant differences between genotypes at the same pH level.

remained stable during fermentation but significantly decreased in baked products made from purple wheat varieties, as measured by the pH differential method. Additionally, research has shown that the type of dietary fibre plays a crucial role in anthocyanin stability (Enaru et al., 2021). When various types of dietary fibres were added, β-glucan exhibited the highest total antioxidant capacity throughout the entire storage period (Pieczykolan & Kurek, 2019).

3.4.3 HPLC Analysis

Anthocyanin peaks were only detectable at 511 nm and 280 nm in HB21148. However, these peaks were difficult to identify in the prepared tortillas. To improve detection, raw barley samples, which had the highest concentration, were spiked into each treatment. Unfortunately, the sample concentrations remained below the range of the calibration curve despite trying various extraction methods and various methods and concentration techniques. As an alternative approach, two anthocyanin standards were added to CDC Rattan with a known concentration of 200 mg/kg. CDC Rattan was chosen for this experiment due to its relatively simple profile of compounds observed at 280nm and high β-glucan content, allowing us to investigate the stability of anthocyanins and dietary fibres using FT-IR analysis.

During the extraction, a red colour was observed in the extract of raw CDC Rattan with added anthocyanins. A similar colour appeared in the pH 3 tortilla dough extract (Appendices S1), but it was no longer visible after cooking. HPLC analysis detected peaks only in the raw sample and the pH 3 dough (Appendices S2 to S4), indicating the presence of anthocyanins in these samples.

The results on quantification and identification of phenolic acids at 280nm are presented in Table 3.1. Notably, catechin was exclusive to purple genotypes HB21148 and HB21147, where it was the dominant phenolic compound. In HB21147, the catechin content in tortilla at pH 5

(65.4±1.6 mg/kg) was 4.5 times higher than in its raw form (22.3±0.5 mg/kg). Both HB21148 and HB21147 showed no significant changes, except at pH 3, where concentrations decreased to 45.3±14.3 and 49.0±0.8 mg/kg, respectively. m-Coumaric acid was detected at both pH 5 and pH 7 in HB21148 (1.63±0.18 mg/kg and 1.81±0.02 mg/kg, respectively), HB21147 (2.144±0.12 mg/kg and 1.56±0.11 mg/kg, respectively) and Roseland (1.62±0.17 mg/kg and 1.82±0.00 mg/kg, respectively). In contrast, CDC Rattan only showed m-coumaric acid at pH 7 (2.16±0.24 mg/kg). These results suggest that m-coumaric acid is more stable in barley tortillas prepared at neutral pH (pH7) compared to acidic conditions. Gentisic acid was detected in HB21147 and CDC Rattan at pH 5, with concentrations of 1.71±0.04 and 1.98±0.03 mg/kg, respectively. Moreover, 3,4-dihydroxybenzaldehyde was found exclusively at pH 3 in Roseland and HB21147 at1.9±0.5 and 3.4±0.3 mg/kg, respectively. 4-Hydroxybenzaldehyde was present in Roseland and CDC McGwire at pH 3, with identical concentrations reported for both . Notably, ellagic and cinnamic acids were found in significantly higher concentrations in raw Roseland (13.4±0.5 and 13.5±0.5, respectively) compared to other treatments, suggesting these phenolic acids may be more sensitive to pH changes and heat than catechin during tortilla production. Quercetin was detected in all the genotypes at pH 1, but quantifiable amounts were only found in HB21148, CDC McGwire and Roseland with regular β-glucan concentrations, at 1.5±0.0, 1.4±0.0 and 1.4±0.0 mg/kg, respectively. The HPLC was able to detect some compounds but the concentration was below the quantification limit (BQL), therefore they were not quantified.

Comparing these findings to existing research, the catechin content in pH 7 tortillas of HB21148 was twenty times higher than that reported in raw purple barley varieties by Jin et al. (2022), Whereas Dang et al. (2022) found half the catechin content in similar tortillas. Notably, p-coumaric acid levels were higher in both HB21147 and CDC Rattan at pH 5 and 7 compared to

concentrations reported during physiological maturity and harvest stages of purple barley growth (Suriano et al., 2018). Ellagic acid was detected in almost all genotypes, supporting the findings that it is a major metabolite in barley, as reported by Eid et al. (2024).

Table 3.1. Polyphenol Compounds (mg/kg) in Barley Genotypes

Genotype	Treatment	Catechin	3,4 Dihydroxy benzaldehyde	Gentisic acid	p-Hydroxybenzoic acid	4-Hydroxybenz-aldehyde	p-Coumaric acid	m-Coumaric acid
HB21148	pH 1	45.6±0.9 bA						
	pH 3	45.3±14.3 bA						
	pH 5	64.4±1.6 aA						1.6±0.2aB
	pH 7	65.9±0.0aA			2.1±0.0aB			1.8±0.0aA
	Raw	22.3±0.5cA			2.4±0.8aB			
HB21147	pH 1	37.5±2.5dB						
	pH 3	49.0±0.8bA	1.9±0.5B					
	pH 5	52.2±2.4aB		1.7±0.0B			1.7±0.0aA	2.1±0.1aA
	pH 7	44.8±1.1cB		.			1.6±0.0aB	1.6±0.1bB
	Raw	11.2±0.1eB						
CDC McGwire	pH 1							
	pH 3							
	pH 5							
	pH 7							2.2±0.2A
	Raw				2.2±0.1B	3.8±0.1B		
CDC Rattan	pH 1							
	pH 3							
	pH 5			2.0±0.0A				
	pH 7				2.3±0.0bA			2.2±0.2A
	Raw				7.5±0.3aA			
Roseland	pH 1							
	pH 3		3.4±0.3A				1.7±0.1aA	
	pH 5				4.9±2.3a		1.8±0.0aA	1.6±0.2aB
	pH 7				1.9±0.1aB			1.8±0.0aA
	Raw					5.3±0.4A		

Table 3.1. cont.

Genotype	Treatment	o-Coumaric acid	Chlorogenic acid	Naringin	Ellagic acid	trans Cinnamic acid	Quercitin dihydrate
HB21148	pH 1	1.4±0.0	2.3±0.4aA		2.4±1.2bA	3.3±0.8bA	1.5±0.0A
	pH 3				1.4±0.0cA	3.0±0.2bA	BQL
	pH 5	1.4±0.0		1.7±0.2aA		1.8±0.1cA	
	pH 7	1.4±0.0	1.4±0.0c	1.9±0.7a	2.1±0.4b		BQL
	Raw	1.4±0.0	1.6±0.0b	1.9±0.1a	5.9±0.5aB	6.7±0.1aB	BQL
HB21147	pH 1	BQL	BQL		1.5±0.1A	1.6±0.2bB	BQL
	pH 3	1.7±0.2	1.7±0.3a			2.3±0.1aB	1.6±0.2a
	pH 5	1.5±0.0		1.9±0.7aA	BQL	2.7±0.2aA	1.5±0.0a
	pH 7	1.6±0.1	1.6±0.2a			2.4±0.1a	1.5±0.0aA
	Raw	BQL	1.4±0.0a	1.4±0.0a		2.4±0.7abD	BQL
CDC McGwire	pH 1				BQL	BQL	1.4±0.0B
	pH 3	1.5±0.1			BQL	BQL	
	pH 5	BQL			BQL	BQL	
	pH 7	1.5±0.1	BQL		BQL	BQL	BQL
	Raw	BQL	1.5±0.1	BQL		5.0±1.0C	
CDC Rattan	pH 1		BQL		BQL		BQL
	pH 3				1.5±0.0aA	BQL	BQL
	pH 5	1.5±0.2			1.5±0.0aA	BQL	BQL
	pH 7	1.8±0.0	BQL		BQL	BQL	BQL
	Raw		1.6±0.3b				
Roseland	pH 1	1.4±0.1	1.6±0.2bB		BQL	BQL	1.4±0.0aB
	pH 3					1.6±0.1bC	
	pH 5		1.7±0.0b	1.8±0.0	1.4±0.0bB	BQL	BQL
	pH 7	1.7±0.0			BQL	BQL	1.4±0.0aB
	Raw	1.8±0.1	2.6±0.1a		13.4±0.5aA	13.5±0.5aA	

BQL - Below Quantification Limit. Different lowercase letters indicate significant differences within the same genotype. Different uppercase letters signify significant differences between genotypes under the same treatment. The results are presented as mean ± SD; n=3.

The polyphenol identification used the same extraction method as for the anthocyanins, ensuring consistent conditions across analyses to isolate the effects of different treatments.

3.4.4 Antioxidant Activity

Figure 3.3 shows the antioxidant activity (AA) of the raw barley and barley tortillas as measured by the FRAP assay. HB21148 and Roseland exhibited similar AA across most treatments, except at pH 3, where HB21148 had higher activity (2181±47 µg/g DW Tx eq) compared to Roseland (1774±55 µg/g DW Tx eq). CDC McGwire consistently showed the lowest AA in all the treatments, followed by CDC Rattan and Roseland.

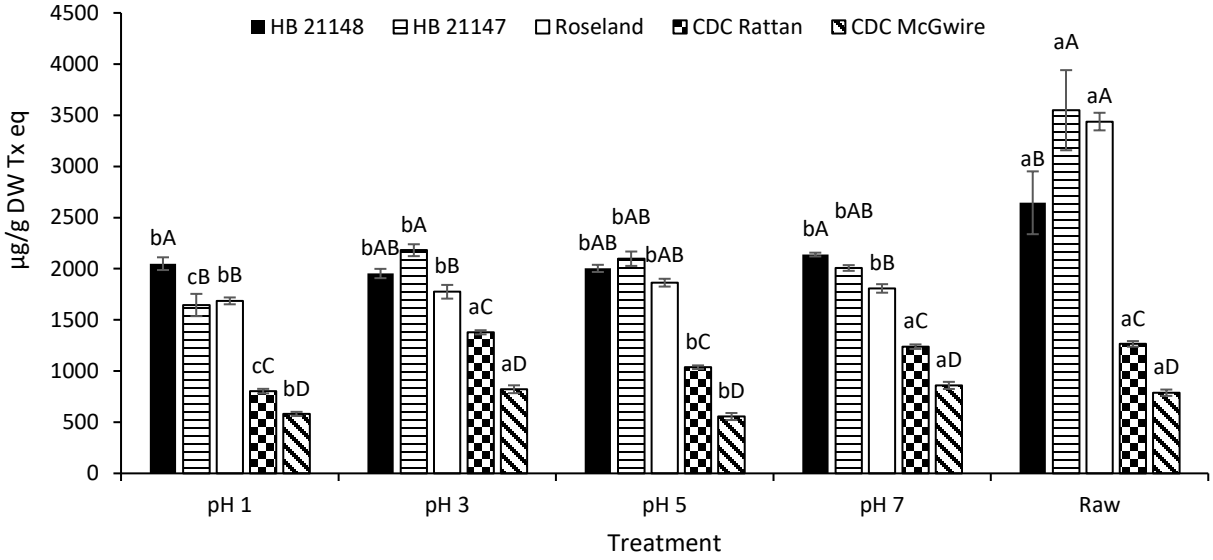

Figure 3.3. FRAP results. The results are presented as mean ± SD; n=4. Different lowercase letters represent significant differences within the genotype. Different uppercase letters represent significant differences between genotypes at the same pH levels. The assay was analyzed using Wilcoxson's test.

Figure 3.4 shows the AA of raw barley and barley tortillas as measured by the ABTS assay. HB21148 and CDC McGwire had similar AA at pH 1, with values of 7042±241 and 6122±816 µg/g DW Tx eq, respectively. Notably, significant differences were only observed in the raw forms of HB21147 (7322±364 µg/g DW Tx eq) and Roseland (8642±305 µg/g DW Tx eq) across the

various treatments (Figure 3.4). In HB21148, pH 1 tortillas showed significantly lower AA within

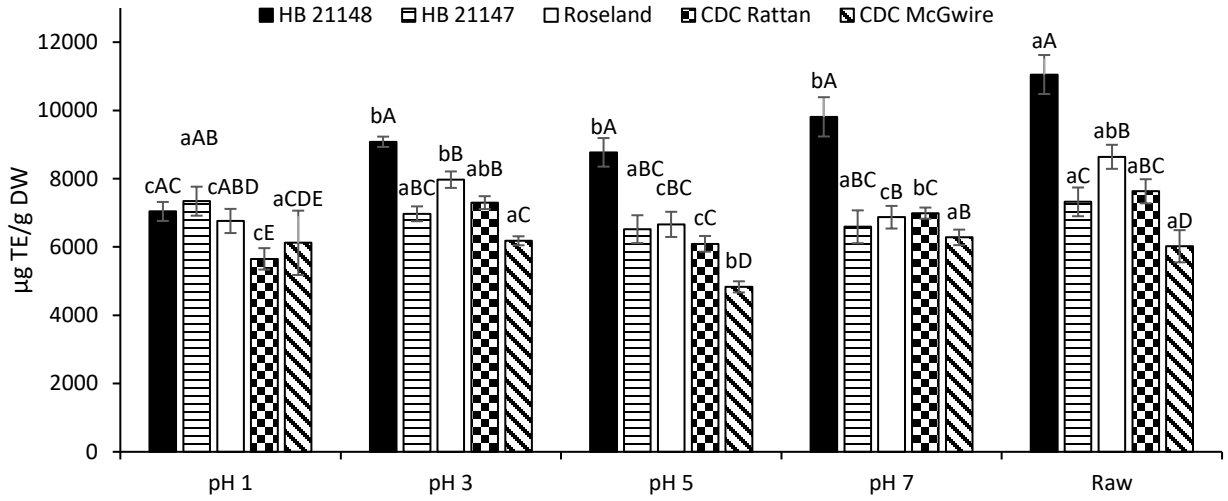

Figure 3.4. ABTS results. The results are presented as mean ± SD; n=4. Different lowercase letters represent significant differences within the genotype. Different uppercase letters represent significant differences between genotypes at the same pH levels.

the genotype (7042±240 µg/g DW Tx eq) (Figure 3.4). In contrast, the AA of HB21147 remained unaffected by treatment when measured using the ABTS method. For most genotypes, pH 3 tortillas and raw barley had similar AA, except for HB21148, which showed distinct values of 9082±130 µg/g DW Tx eq for pH 3 tortilla and 11052±494 µg/g DW Tx eq for raw barley (Figure 3.4). HB21147 and CDC Rattan exhibited comparable AA across treatments, with no significant differences (Figure 3.4). These findings align with Zhang et al. (2021), who reported similar trends in ABTS and FRAP assays, with purified anthocyanin extract showing comparable activity to the positive control.

Figure 3.5 illustrates the AA of raw barley and barley tortillas using the DPPH assay. According to the DPPH results, HB21148, HB21147, and Roseland were not significantly different in AA at pH 1, pH 3, and pH 5 (Figure 3.5). At pH 7, HB21148 (941±6 µg/g DW Tx eq) and HB21147 (935±27 µg/g DW Tx eq) had similar AA, which was higher than that of CDC McGwire and CDC Rattan (488±9 and 532±20 µg/g DW Tx eq, respectively). In their raw forms, CDC

Rattan and CDC McGwire exhibited the lowest AA (442±10 and 449±12 µg/g DW Tx eq, respectively) (Figure 3.5). Notably, the treatments positively impacted the AA of CDC Rattan and

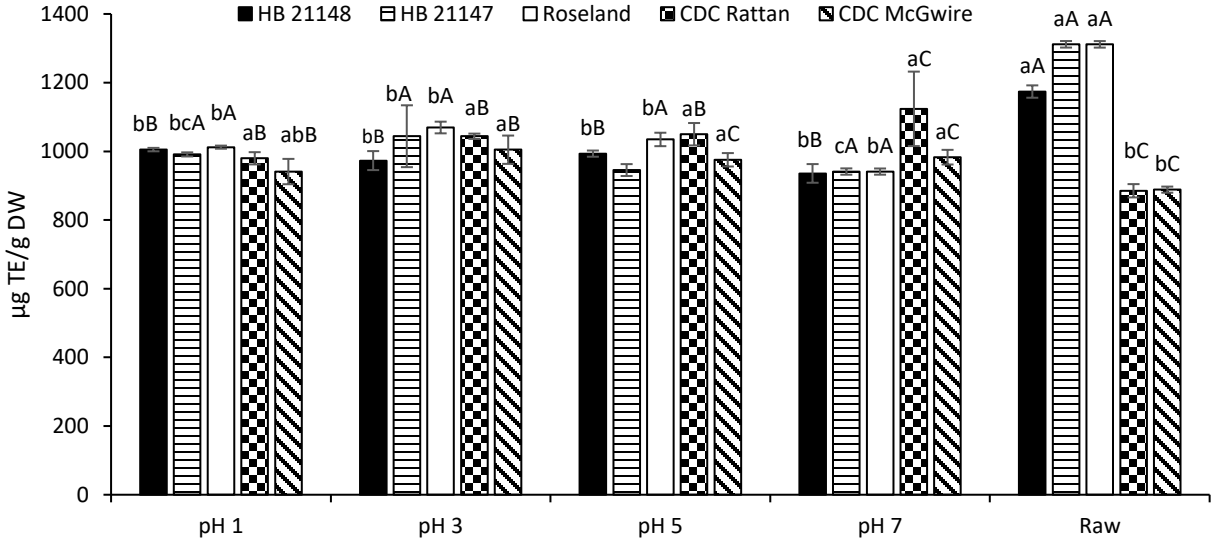

Figure 3.5. DPPH results. The results are presented as mean ± SD; n=4. Different lowercase letters represent significant differences within the genotype. Different uppercase letters represent significant differences between genotypes at the same pH levels.

CDC McGwire whereas Roseland, HB21147 and HB21148 showed no such effect (Figure 3.5).

In the study, barley colour did not influence the DPPH and ABTS AA assays, consistent with the findings of Deng et al. (2021). However, their results primarily showed similarities in these assays for the white and yellow varieties. Baking has been shown to increase AA against ABTS and DPPH, with yellow and purple wheat varieties exhibiting significantly higher results in different parts of the bread (Yu & Beta, 2015). Guan & Zhong (2015) attempted to improve anthocyanin stability during heating to 126°C at pH 5 by using gum Arabic. The AA in FRAP, ABTS, and DPPH assays were significantly enhanced. This suggests that incorporating polysaccharides into food products can stabilize anthocyanins at pH 5. Additionally, previous research has reported that metal ions can enhance anthocyanin stability. However, the effect depends on the monomeric anthocyanin and pH level (Xiong et al., 2006), as observed in extracting

anthocyanins from β-glucan gel. Cyanidin-3-O-rutinoside and C-3-G displayed a higher relative content of anthocyanins in the prepared glucan gel mix compared to delphinidin-3-O-glucoside and delphinidin-3-O-rutinoside (Xiong et al., 2006). Additionally, when the gel extract was heated to 60-100°C at pH below 4.5, it exhibited similar AA in the FRAP assay across all temperatures (Xiong et al., 2006).

The interaction between polyphenols, anthocyanins and dietary fibres has been extensively discussed (Angulo-López et al., 2022). The dietary fibres present in HB21148 are likely responsible for the elevated TPC, thus contributing to increased AA (González-Aguilar, Blancas-Benítez, & Sáyago-Ayerdi, 2017). These dietary fibres form hydrogen bonds with polyphenols potentially protecting them during processing (Maréchal, 2007). Notably, the anthocyanins in CDC Rattan were not detrimental. According to Bohn (2014), dietary fibres undergo fermentation during digestion, releasing the polyphenols and enhancing their stability, which can amplify their antioxidant effects in the body, a benefit that may not be fully captured by laboratory methods.

3.3.1 Cell Viability

Figure 3.6 shows the effects of HB21148 and CDC Rattan+ACN extracts on Caco-2 cell viability. As HB21148 concentrations increased, cell viability decreased (Figure 3.6 A). At 25 µg/ml of HB21148, the cell viability was similar to that of the control group. In contrast, cells incubated with HB21148 (50, 100, 200, 400, and 800 µg/ml) showed a significantly lower viability (80.4%, 77.4%, 77.3%, 67.7% and 61.6%, respectively) compared to the control. This significant decrease in cell viability suggests a cytotoxic effect of HB21148 at high concentrations. Caco-2 cells treated with CDC Rattan+ACN (25 µg/ml – 800 µg/ml) showed a similar cell viability trend to HB21148, with a notable decline in cell survival at concentrations of 100 – 800 µg/ml (Figure

3.6 B). The results indicate that both HB21148 at 25 µg/ml and CDC Rattan+ACN at 25 and 50 µg/ml) were non-cytotoxic to Caco-2 cells.

Cell viability decreased as sample concentration increased, a trend consistent with findings by Cui et al. (2020) in flavonoid-enriched buckwheat. Their study showed comparable cell viability to non-enriched samples at 100 µg/ml, but lower viability in non-enriched samples at 200 µg/ml. Additionally, they found that standard flavonoids reduced cell viability in a concentration-dependent manner (Cui et al., 2020).

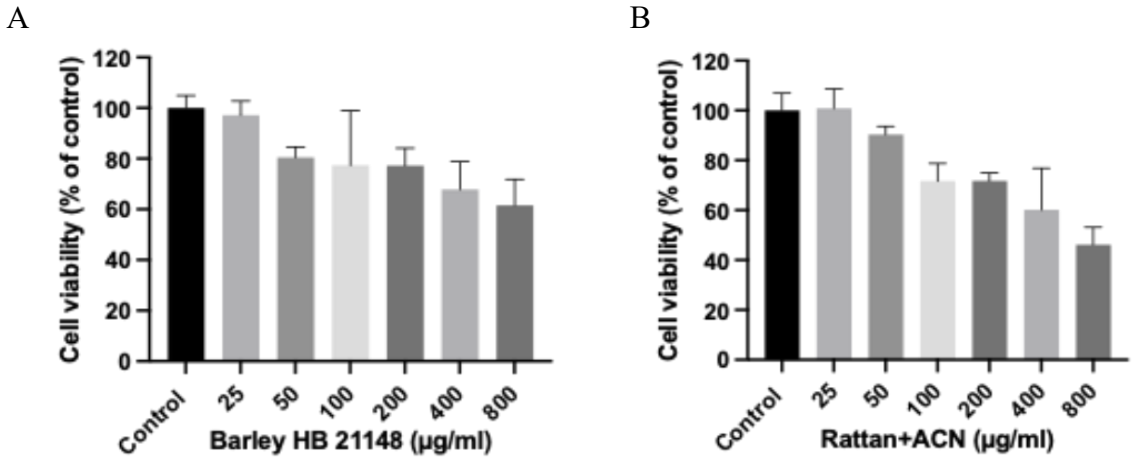

Figure 3.6 Caco-2 cell viability. (A) Cells were incubated with different concentrations of Barley HB21148 and (B) cells with varying concentrations of Barley CDC Rattan+anthocyanin (ACN) for 24h. Results are presented as a percentage of control cells, with each bar representing the mean ± SD (n=6).

3.3.2 Intracellular Antioxidant Activity of Barley Samples against AAPH-induced Oxidation

To assess the protective effects of barley extracts against AAPH-induced oxidative stress, Caco-2 cells were pretreated with CDC Rattan+ACN and HB21148 at various concentrations using DCFH-CA (2.5 µM) as a probe. The results are presented in Figure 3.7.

CDC Rattan supplemented with anthocyanins (Rattan+ACN) exhibited intracellular AA units of 25.3, 26.3, and 32.9 at concentrations of 50 µg/ml, 100 µg/ml, and 200 µg/ml, respectively. In comparison, the CAA units of HB21148 at the same concentrations were 21.6, 29.9, and 19.2, respectively. The results showed an increasing trend in CDC Rattan+ACN CAA units, suggesting that CDC Rattan+ACN protected Caco-2 cells in a dose-dependent manner against AAPH-induced oxidation. Although HB21148 exhibited CAA at 50, 100, and 200 µg/ml against AAPH, a notable decrease in CAA at 200 µg/ml was observed. This suggests that the protective effect of HB21148 was not dose-dependent and that 200 µg/ml may not be an optimal concentration for preventing AAPH-induced oxidation in Caco-2 cells. Comparing the two barley samples (CDC Rattan+ACN and HB21148) CAA at the same concentrations, only CDC Rattan+ACN at 200 µg/ml displayed a significantly higher CAA than HB21148, potentially due to the antioxidant properties of supplemented anthocyanins. These findings align with studies showing higher CAA in free phenolics compared to bound forms (Xiong et al., 2021). Gaxiola-Cuevas et al. (2017) also found that while white and blue maize had similar CAA from free and bound phenolics, blue maize had significantly higher total CAA, likely due to its higher anthocyanin content.

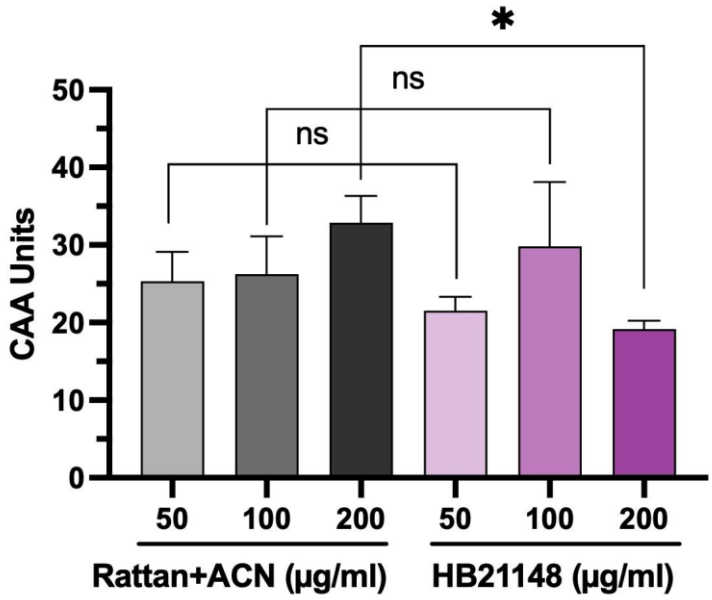

Figure 3.7. Cellular antioxidant activity of CDC Rattan+ACN and HB21148 extracts against AAPH-induced oxidation in Caco-2 cells. Data are expressed as mean ± SD (n=3). * Significant difference at $p<0.05$; ns= not significant

3.5 CONCLUSIONS

This study found that pH treatment had varying effects on TPC across barley genotypes. In HB21147, TPC remained unaffected by treatment, while CDC McGwire showed significantly higher TPC at pH 5 treatment (10971±1837 µg GAE eq/g). In contrast, untreated HB21148 had the highest TPC (14066±1238 µg GAE eq/g). Both HB21148 and CDC McGwire had a significantly higher TAC in the untreated samples, however, the treatment did not affect the TAC. Analysis of specific phenolic compounds revealed catechin only in the purple genotypes (HB21147 and HB21148), with significantly lower concentrations in raw samples. m-Coumaric acid was present in all the genotypes at pH 7.

Using the FRAP method, HB21148 (2644±307 µg/g DW Tx eq), HB2117 (3549± 391 µg/g DW Tx eq), and Roseland (3438±85 µg/g DW Tx eq) had significantly higher AA in the raw sample compared to treated samples. In CDC Rattan, AA values were similar in raw samples and

tortillas at pH 3 and pH 7. With the ABTS assay, CDC Rattan (7299±189 µg/g DW Tx eq) and Roseland (7972±241 µg/g DW Tx eq) had significantly higher AA at pH 3. In contrast, the DPPH assay revealed significantly lower AA in raw CDC Rattan (855±19 µg/g DW Tx eq) compared to all treated samples.

The results obtained from CAA and *in vitro* methods suggest that anthocyanins are key contributors to antioxidant activity. However, other compounds such as dietary fibres and various polyphenols likely play significant roles as well, indicating a potential synergistic effect in overall antioxidant activity.

The study shows that pH treatment significantly impacts the phenolic composition, colour, and antioxidant activity of barley. While antioxidant activity appears to be pH-dependent, a direct correlation with phenolic composition has not been established. Notably, this research is one of the first to investigate pH effects specifically in barley, differing from existing studies that often focus on by-products like peels. The findings, combined with existing literature and the simplicity of our tortilla composition, suggest that the proposed mechanism may underlie the unexpected observed changes.

The relationship between pH and its effects on anthocyanins, phenolic compounds, β-glucans, and heat is complex and warrants further investigation. However, the findings suggest that a pH level of 3 may be optimal for enhancing antioxidant activity.

Chapter 4: GENERAL CONCLUSIONS and RECOMMENDATIONS

4.1 General Conclusions

This study explored the potential of optimizing pH levels to improve anthocyanin stability during tortilla production. The hypothesis that the lowest pH would yield the highest TAC, TPC, and antioxidant activity was partially confirmed. Specifically, pH 3 consistently produced the best antioxidant results across all three assays (DPPH, ABTS, and FRAP) for all genotypes. The cell experiment suggests this may be due to the intermolecular forces between polyphenol and other bioactive compounds, particularly β-glucans. The treatment had no significant effect on thickness in all barley genotypes except CDC Rattan. For CDC Rattan, the only notable differences in thickness were observed at pH levels 1 and 7, which measured 3.3 ± 0.3 mm and 3.6 ± 0.3 mm, respectively. The analysis of polyphenols yielded valuable findings, particularly regarding catechin, which was exclusively detected in the purple genotypes. Notably, the treatment significantly increased catechin concentrations compared to the raw forms. A striking example was HB21147 at pH 5, where the catechin content of the tortilla surged to nearly five times that of its raw counterpart. Interestingly, certain compounds - including 3,4 dihydroxybenzaldehyde, gentisic, p-coumaric, and m-coumaric acids - were only detected after the treatment and were absent in the raw form. Conversely, the levels of trans-cinnamic and ellagic acids decreased across all treatments for the HB21147, CDC McGwire, and Roseland genotypes.

The outcome is a colourful and appealing tortilla with potential enhanced health benefits, achieved by using pH 3 and the HB21148 genotype. This approach may maximize the health potential of β-glucan, boasting higher anthocyanin content and improved antioxidant activity.

The implications of this research are significant for both the food industry and plant science. Developing crops rich in naturally occurring anthocyanins, polyphenols, and dietary fibers can substantially increase the health benefits of food products. Typically, adding preservatives extends shelf life and ensures product safety. However, this study demonstrates that leveraging the natural composition of barley grain can be accomplished through a simple method, eliminating the need for additives.

4.2 Limitations and Recommendations

The extensive number of samples and treatments in each experiment led to a multitude of comparisons. To address this challenge, the statistical method employed in this study effectively controlled the false-positive rate. Nevertheless, to pinpoint genuinely significant comparisons, a larger sample size (exceeding 10) is required for each sample in every experiment. Unfortunately, increasing the number of replications would substantially prolong the duration of each experiment. Another avenue worth exploring is the examination of parent barley lines to ascertain whether cross-breeding has induced changes. This investigation would offer valuable and innovative insights into the impact of this process on antioxidant activity and the physical properties of barley tortillas. Collaborating with the chemistry department to utilize MS/MS technology was a valuable experience. However, several challenges arose during the identification process, making it more difficult and time-consuming. The extraction method plays a crucial role in shaping the results. However, due to the complexity of our study, which involved 25 samples (4 pH treatments and raw samples for five barley genotypes), we couldn't identify a single "ideal extraction method." Selecting a method that aligned with our hypothesis could have introduced bias, so we opted for a more neutral approach.

While analyzing physical parameters, chemical composition, and antioxidant activity provides valuable insights, it may not be enough to capture the full complexity of this research. To address this, we recommend the following avenues for future studies:

1. Employing FT-IR spectroscopy to analyze raw flour, dough, and tortillas can reveal the chemical transformations that occur during processing.

2. Utilizing scanning electron microscopy (SEM) to examine the microstructure of all samples can provide a deeper understanding of the tortillas' physical properties.

3. Conducting sensory panels can help determine whether pH treatments result in noticeable differences and preferences, offering valuable information on the tortillas' overall acceptability.

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Appendices

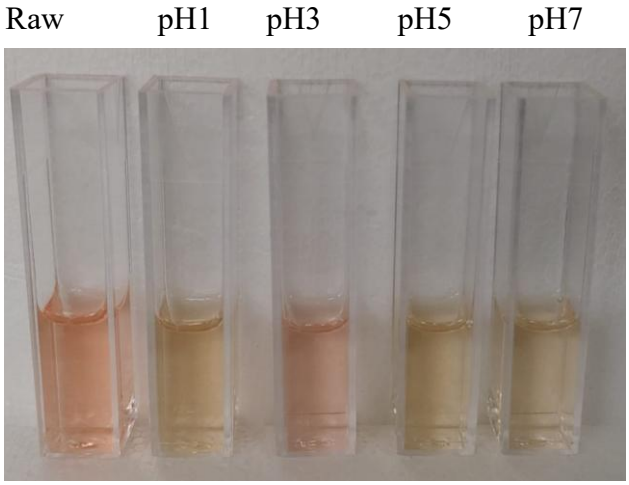

Figure S1. Dough extract of CDC Rattan enriched with anthocyanin standard.

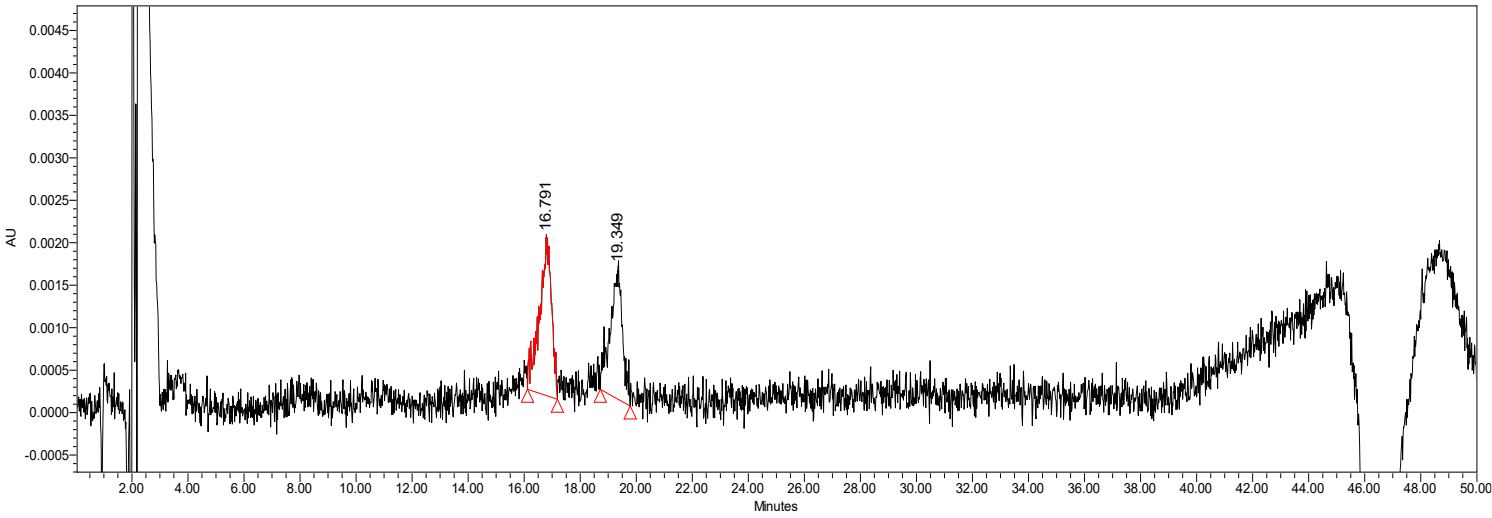

Figure S2. RAW flour CDC Rattan with anthocyanins chromatogram.

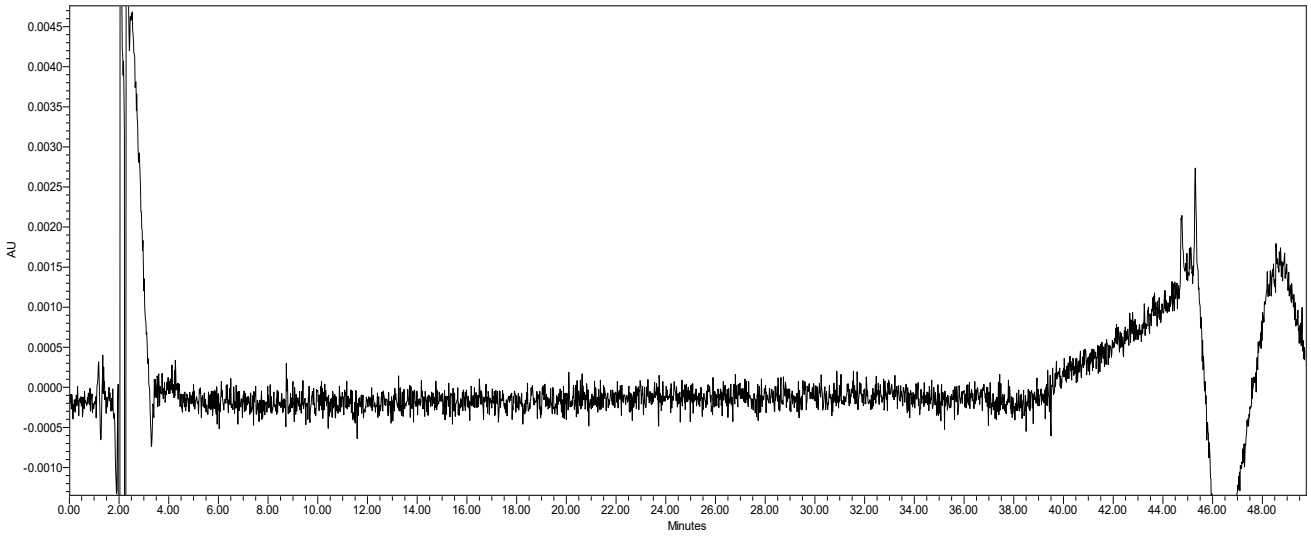

Figure S3 CDC Rattan with anthocyanins tortilla at pH 3 chromatogram.

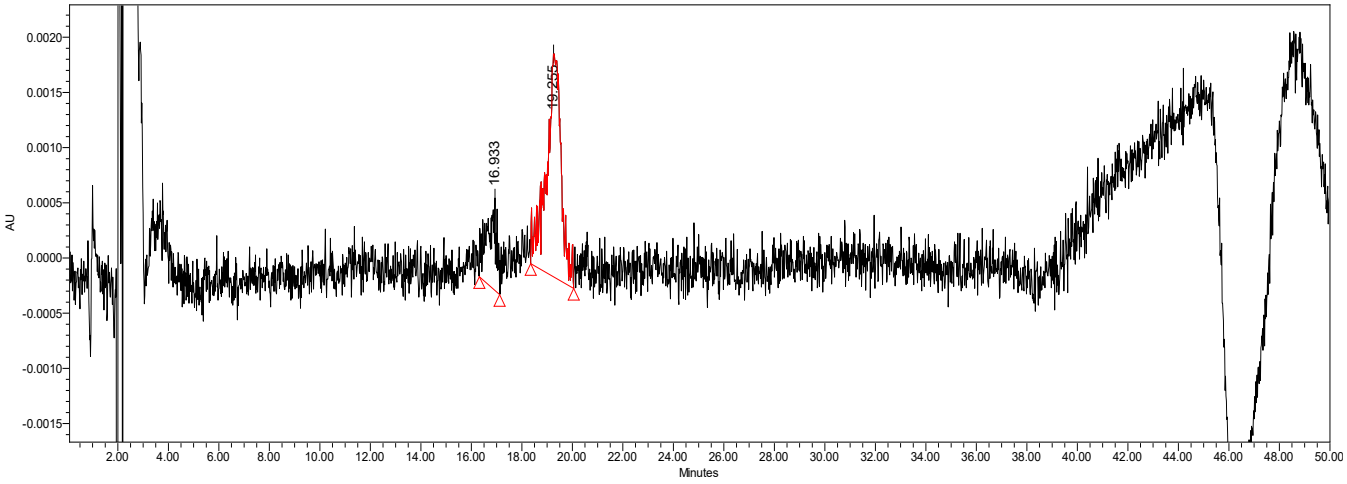

Figure S4. CDC rattan with anthocyanins dough at pH 3 chromatogram.

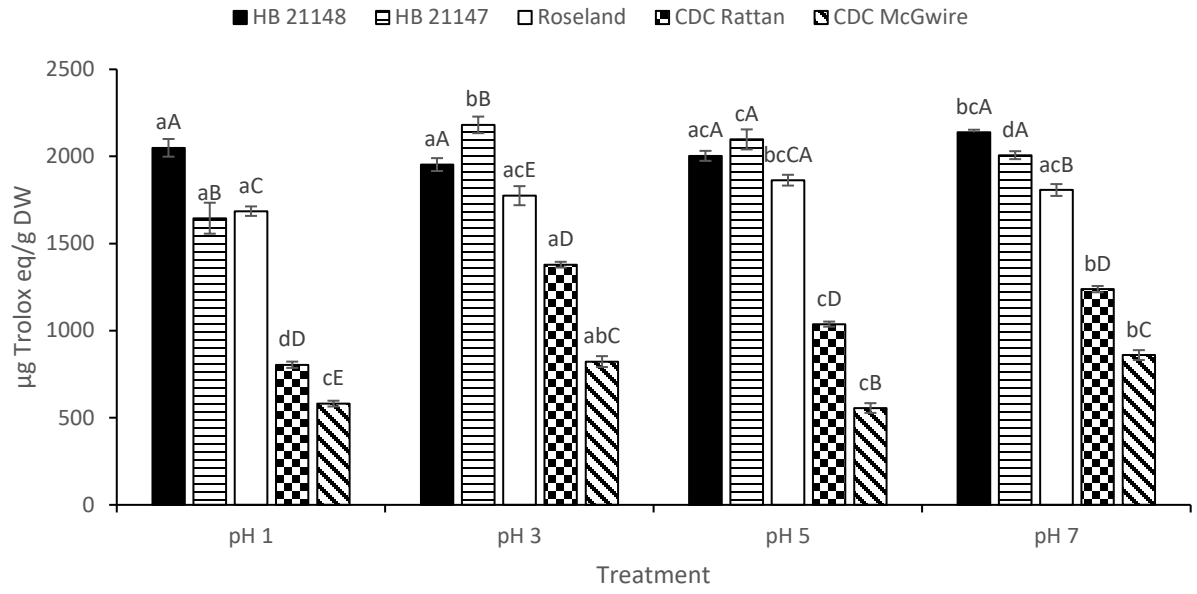

Figure S5- FRAP method without raw sample

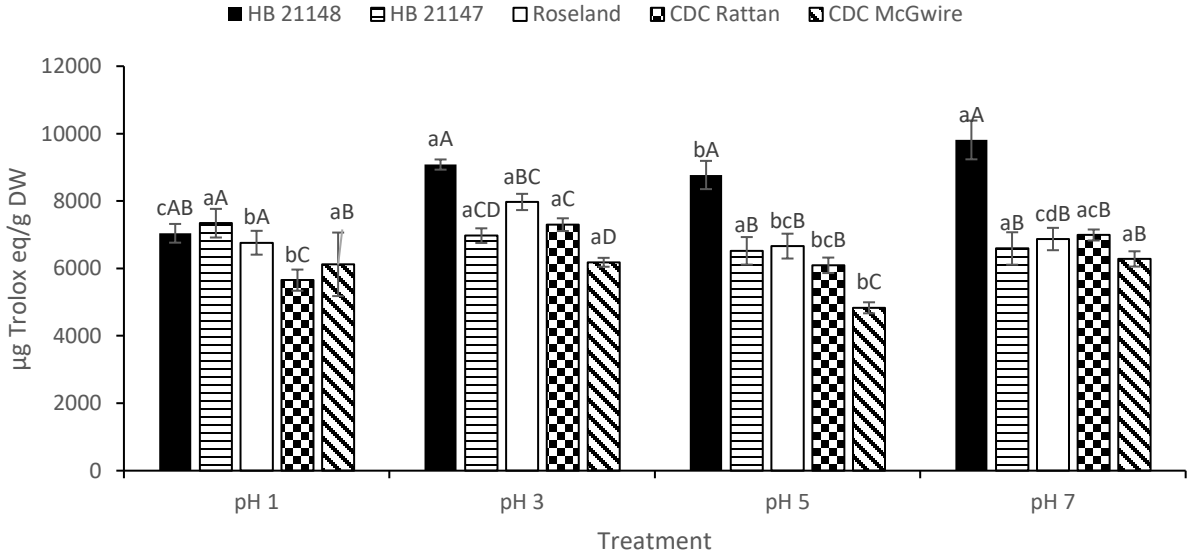

Figure S6- ABTS method without raw sample

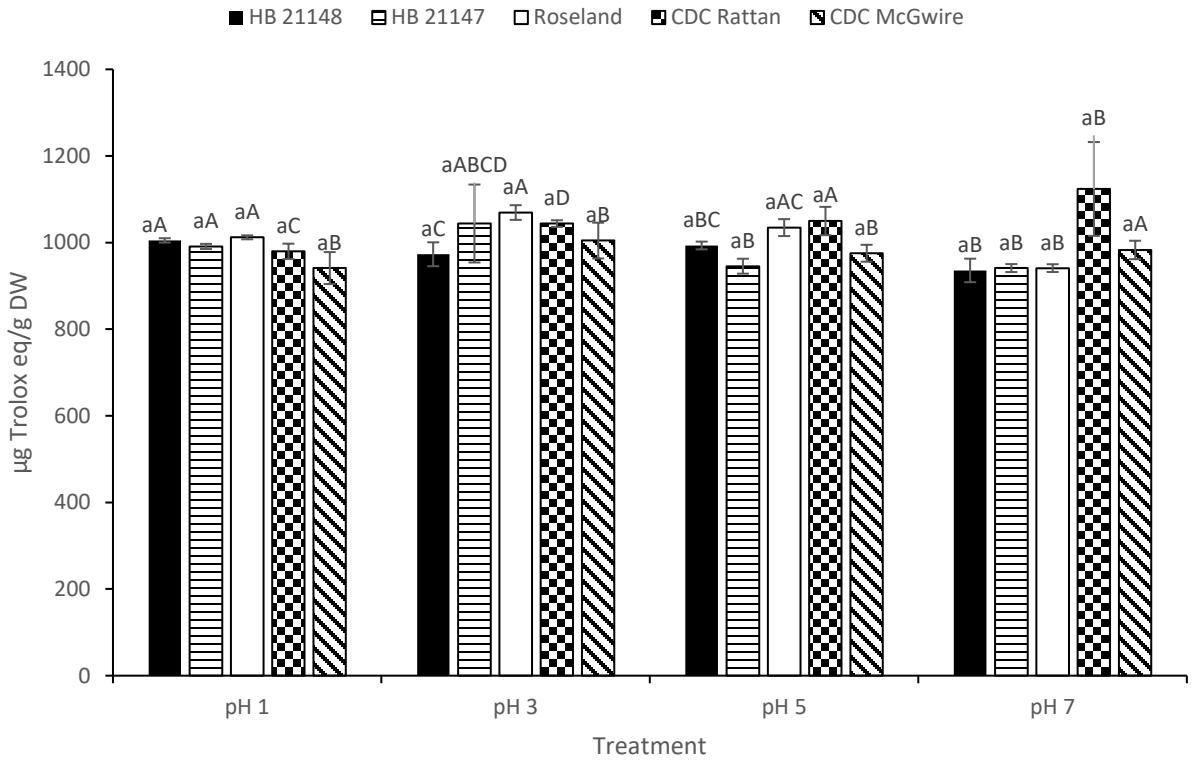

Figure S7- DPPH method without raw sample

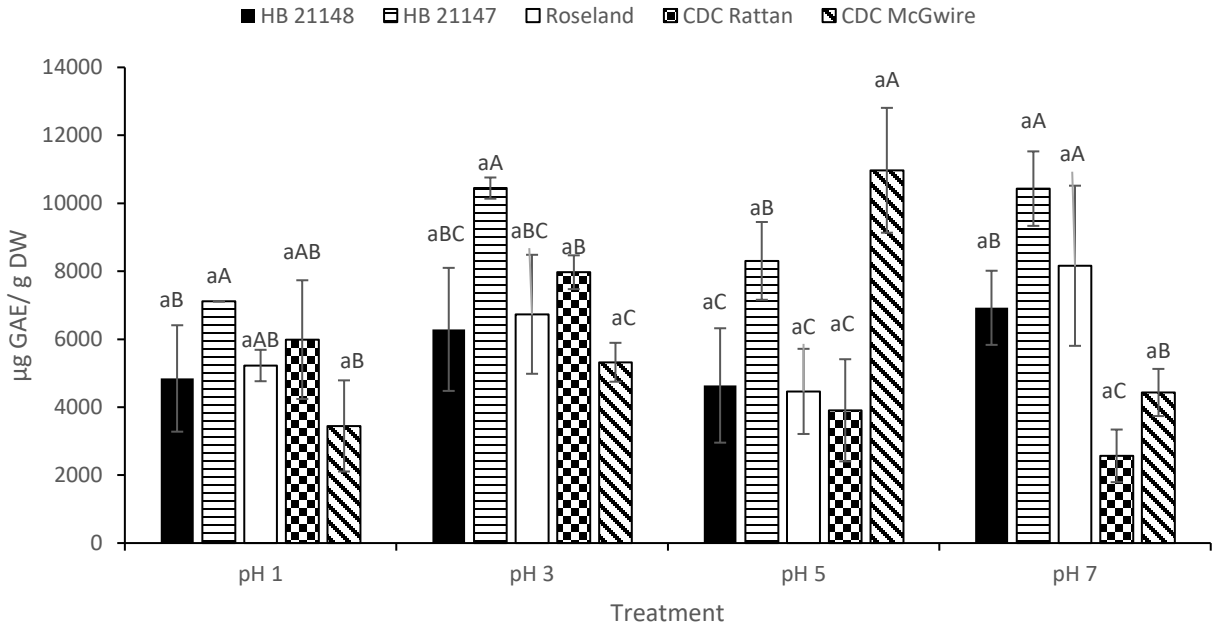

Figure S8- TPC method without raw sample

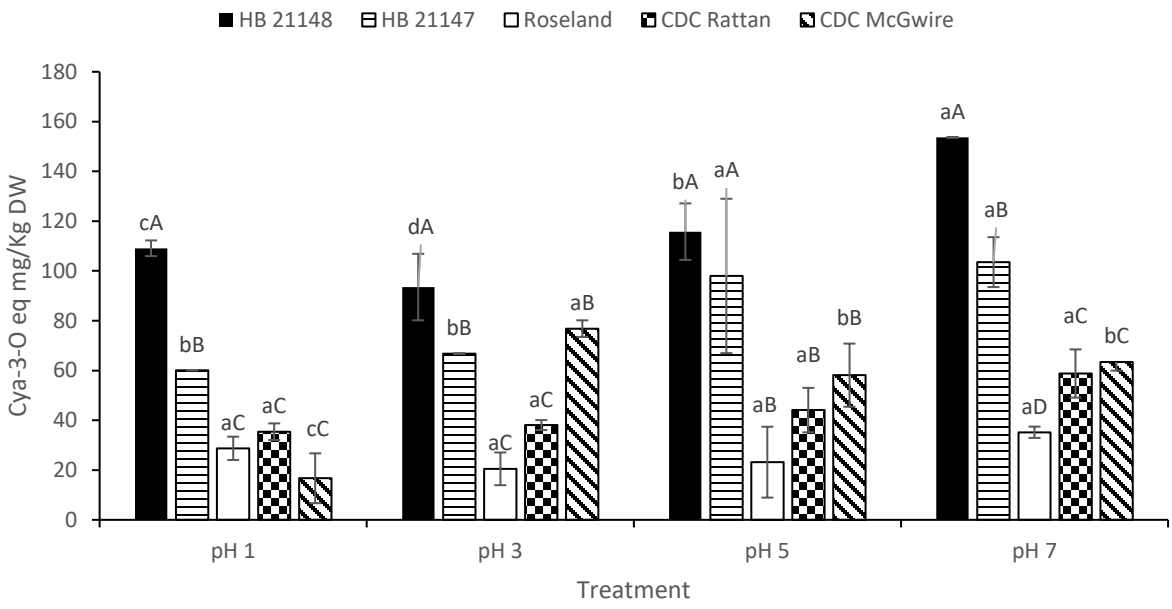

Figure S9- TAC method without raw sample