

**Proanthocyanidins, Anthocyanins and Phenolic Acids in Food Barleys of  
Diverse Origin**

**by**

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## ABSTRACT

Phytochemicals found in grains complement those found in fruits and vegetables. These phytochemicals, though minor compounds, contribute to the antioxidant properties which are related to the health benefits associated with the consumption of whole grain. In this thesis project, nine barley genotypes of diverse origin namely CI2230 from Nepal, CI1248 from Israel, 3 Peruvian genotypes; Peru 3, Peru 16 and Peru 35, Hokuto Hadaka from Japan, EX116; a cross between Moroccan and Canadian genotype, EX83; a cross between two Canadian genotypes and EX127; a cross between Canadian and German genotypes were studied. The genotypes were categorized based on appearance into purple, black and yellow grains. Phenolic acids and flavonoids were identified and quantified in these diverse genotypes using HPLC-ESI-MS analysis. The main classes of dietary flavonoids studied in the barleys were anthocyanins and flavan-3-ols. Phenolic acids were identified and quantified (*p*-coumaric, ferulic, sinapic, caffeic, vanillic). Three ferulic acid dehydrodimers (8-0-4' DFA, 8-5' benzofuran form and 5-5' DFA) were also identified. The most abundant dimeric flavan-3-ols were procyanidins B3 and prodelphinidin B3. The monomeric unit, (+)-catechin, was the most abundant while catechin glucoside (m/z 451) was also identified. Among the Peruvian genotypes, Peru 16 and Peru 35 exhibited relatively high levels of total PA content. Total phenolic content and antioxidant activities of methanolic, acetone and alkali hydrolyzed extracts of the nine barley genotypes was determined by the Folin- Ciocalteu assay, DPPH radical scavenging assay and oxygen radical absorbance capacity (ORAC assay). The acetone extract exhibited the highest antioxidant capacity using all the methods of analysis. Furthermore, dark colored grains were found to exhibit higher contents of phenolic compounds. The phenolic acids, PAs and anthocyanins identified and quantified had significant contribution to the overall antioxidant capacity of the barley whole grain. Four hull-less genotypes namely CI2230, EX127, CI1248 and Peru 35 were further partially sprouted to establish the effects of sprouting on phenolic acid composition. Partial sprouting was observed to significantly increase the soluble conjugated phenolic acids. The barley

genotypes studied were found to contain different quantities of phytochemicals and had high proanthocyanidin content thereby rendering them as alternative sources of antioxidants. Barley sprouts present a possible novel food ingredient with improved properties such as phenolic acid composition and other benefits such as easier incorporation into food products under development.

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## **FOREWARD**

This thesis has been prepared in the manuscript format in adherence with the guidelines established by the Department of Food Science at the University of Manitoba. The Cereal Chemistry journal was the reference style used. This thesis consists of two experimental chapters.

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### ***List of Abbreviations***

a*	Degree of redness
AAPH	2, 2'-azobis (2-amino-propane) dihydrochloride
ABTS	2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt
ACE	Acetone Extract
AUC	Area under the curve
b*	Degree of yellowness
CA	Caffeic acid
CE	Catechin equivalents
CUPRAC	copper reduction assay
DAD	Diode array detector
DFA	Dehydrodimer ferulic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
ET	Electron transfer
FA	Ferulic acid
FAE	Ferulic acid equivalents
FC	Folin-Ciocalteu reagent
FRAP	Ferric reducing antioxidant power
HAT	Hydrogen atom transfer
HPLC	High Performance Liquid Chromatography
HPLC-ESI-MS	High performance liquid chromatography coupled with electrospray ionization mass spectroscopy
HPLC-MS	performance liquid chromatography coupled with mass spectroscopy
L	Degree of lightness
LSD	Least significant difference
MET	Methanolic Extract
ORAC	Oxygen radical absorbance capacity
PA	Proanthocyanidins
<i>p</i> -CA	<i>p</i> -Coumaric acid
PCL	Photochemiluminescence
PDA	Photodiode array detector
Q-TOF	Quadruple/ time of flight
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SA	Sinapic acid
TAC	Total Anthocyanin content
TE	Trolox equivalents
TEAC	Trolox equivalent antioxidant activity
TPC	Total phenolic content
TRAP	Total radical trapping antioxidant parameter
VA	Vanillic Acid

## ***Introduction***

Grains are grown in greater quantities and provide more energy worldwide than any other type of crop. Cereal crops are mostly grasses cultivated for their edible grains or seeds (caryopsis). Barley (*Hordeum vulgare* L.), a member of the grass family *Poaceae* accounts for 15% of world coarse grain use, being second to corn (68%) (Agriculture and Agri-Foods Canada 2005). Barley was one of the first cereal crops to be domesticated. Canada is one of the largest barley producers in the world producing nearly 12 million metric tons (Statistics Canada 2006). There is substantial evidence that portrays the role of barley as a sustaining source of food in the evolution of humans. Archaeologists have found evidence in the Middle East that it was a staple food as far back as 5,000 BC or even earlier. It also ranks fourth among the cereals in worldwide production. There are different varieties of barley grown worldwide and different types are best for different uses. Health benefits of various plants are attributed to the differences between compositions of phytochemicals with different structures found in the plants (Adom et al 2002).

Barley is an important crop for direct human consumption, animal feed and industrial applications. When consumed together, grains contribute phytochemicals that complement those found in fruits and vegetables. Many consumers believe that food plays a significant role in maintaining or improving overall health and that certain food may have health benefits beyond basic nutrition. Furthermore, people are becoming aware that certain foods, because of the presence of bioactive compounds or chemicals, may have positive impact on an individual's health, physical well being and mental state. As a result of the side effects attributed to the consumption of drugs and supplements, the use of "healthy foods" that are "functional" has been

on the rise. Barley and other cereal grains present an opportunity for the production of functional foods due their high amounts of phytochemicals.

Consumption of whole grain is associated with a decrease in incidents of chronic diseases such as cancers, cardiovascular diseases and diabetes. The health benefits associated with barley grain are attributed to the presence of phytochemicals and their antioxidant properties. The recommended food guide servings per day for grain products is 6-7 for females and 8 for males. Health Canada also recommends that at least half of the grain products consumed each day should be whole grain (Health Canada 2007). Truswell et al (2002) reported that there was an inverse relationship between the consumption of whole grains and risk of cardiovascular disease (CVD).

The advantages of obtaining phenolic compounds from cereals rather than fruits and vegetables are that cereals are dry, easy to store and to process into shelf-stable products for a long duration. The current consumer interest in nutrition and health gives room for research in barley and potential benefits in human diet. The task now is to isolate, characterize and quantify these compounds that contribute to the associated health benefits. The objectives of this study were:

- To selectively extract, identify and quantify the major phenolic compounds from nine diverse barley genotypes employed as food, feed and malt
- To determine the antioxidant capacity of the isolated extracts so obtained from the barleys.

- To evaluate the effect of sprouting on free, soluble conjugated and insoluble bound phenolic acids composition of the partially sprouted and non sprouted whole barley grains of diverse origin and
- To evaluate antioxidant activity of partially sprouted and non sprouted whole barley grains of diverse origin.

## ***Chapter 1. Literature review***

### ***1.1 Types of Barley***

Barley can be classified based on physical properties, processing and grain composition. Some of the classes include spring or winter types, two-rowed or six-rowed, hull or hullless, malting or feed use, normal, waxy or high amylose starch, high lysine, high  $\beta$ -glucan, and proanthocyanidin - free. Barley may also be divided by the number of kernel rows in the head. There are two types; two-row barley and six-row barley. Two-row barley has lower protein content than six-row barley but higher enzyme content. High protein barley is best suited for animal feed or malt that has a large adjunct content. Two-row barley is best suited for pure malts (American Malting Barley Association 2005). All the three spikelets attached at each end of the node are all fertile while only the central spikelet is fertile in the two-rowed (Newman and Newman 2008). There are naked and hulled barleys, the hulled barleys being the older forms. In Canada, the barley is either the two-rowed or six-rowed variety that can be used as feed or for malting. Various studies have been done on the phenolic composition and antioxidant activity of hulled and hullless barley varieties (Holtekjolen et al 2006, Quinde-Axtell et al 2006, Verardo et al 2008).

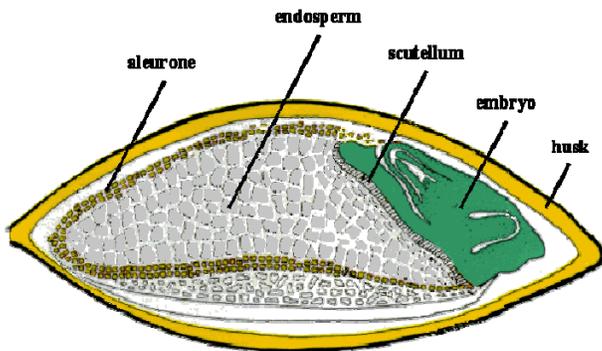
Barley varieties that are proanthocyanidin-free have been developed because proanthocyanidins decrease the digestibility of proteins (MacGregor et al 1993). Furthermore, proanthocyanidin-free varieties are utilized in beer production because proanthocyanidins bind proteins and contribute to haze formation (Holtekjolen et al 2006). Zhou et al (2008) and Hernaz

et al (2001) evaluated antioxidant activities and total phenolic contents of typical malting barley varieties. The phenolic acid contents have been assessed in different malting and feed barleys of U.S., Canadian, and European origin (Zupfer et al 1998). As an anthocyanin-colored cereal, barley exists in black, red, blue and purple varieties (Takashi et al 1955). Kim et al (2007) studied 127 lines of colored barley; however, in this present study barley antho-lines of diverse origin were evaluated for their phytochemical profiles and antioxidant properties.

## 1.2 Structure of Barley

There are several distinct species of barley, although the most commonly cultivated is designated as two-rowed or two-eared barley. In general structure, the barley grain resembles wheat and oats as shown in

**Figure 1.1.** The caryopsis is composed of the pericarp, germ and endosperm while the hull contains the lemma and palea. The barley grains are harvested with the hull intact; however, some cultivars are hullless. The hull makes up 10% of the kernel and the average barley kernel weighs approximately 35 mg (Sidhu et al 2007). The aleurone layer can be blue or white depending on the cultivar. The germ makes up 3% of the kernel and is rich in lipids thereby making the grain susceptible to rancidity.



**Figure 1.1: Structure of the barley grain ([www.crc.dk/flab/the.htm](http://www.crc.dk/flab/the.htm))**

### ***1.3 Composition of Barley***

#### ***1.3.1 Major Constituents***

##### ***1.3.1.1 Starch***

Grains are a source of energy which is stored as starch. The starch in grains accounts for 60-75% of the total grain weight. Starch plays a vital role in the physical properties of different food products including the gelling of pudding, setting of cakes and thickening of gravies. Formation of starch granules occurs in the plastids known as amyloplasts. Like wheat starch, barley starch has both large lenticular granules and small spherical granules (Hoseney 1992). Size, shape and gelatinization temperature of starch varies widely among cereals. The ratio of amylose to amylopectin is usually constant with approximately 23% amylose, however, high amylose and waxy barley starches have been reported (Holtekjolen et al 2006). The starch is mainly found in the endosperm of a mature kernel, however, the distribution is not uniform (MacGregor et al 1993). Amylopectin in barley starch is responsible for the crystallinity found in starch granules.

##### ***1.3.1.2 Non- Starch Polysaccharides***

Barley is a good source of both soluble and insoluble dietary fibre. The dietary fibre helps in maintaining the health of the digestive system. It is the soluble fibre consisting of beta-glucans and pentosans (arabinoxylans) that is responsible for the lowering of the serum cholesterol, postprandial blood glucose and insulin levels in humans (Bengtsson et al 1990).

The major components of the walls of selected tissues of barley grain are arabinoxylans and (1→3), (1→4) β-glucans (Henry 1987). The latter are made up of β-glucosyl residues polymerized through the (1→3) and (1→4) linkages (MacGregor et al 1993). Levels of β-glucans in grain can influence the processing and quality of subsequent products during malting and brewing. Arabinoxylans are found in the husk but are abundant in the walls of the aleurone cells and starchy endosperm (Voragen 1987). This major group of noncellulosic polysaccharides is made up of the pentoses, arabinose and xylose. Other polysaccharides found in the walls include (1→3) - β-glucans, (1→4) β-glucans (cellulose) and glucomannans (MacGregor et al 1993). The dietary fibre contents in cereals will vary depending on component of the grain, cultivar and processing conditions (Sidhu et al 2007). In a study done by Charalampopoulos et al (2002), the total dietary fibre in barley was 10% dry basis. Cereals harvested with hulls intact such as oats, rice and barley have high cellulose content. The pericarp is also rich in cellulose.

### ***1.3.1.3 Proteins***

Cereal proteins can be classified according to functionality namely storage and non storage. The storage proteins account for 50% of the total protein in mature cereal grains and can be used in determining the quality and end use of the grain (Shewry et al 2001). The stored protein is used by future seedlings. Cereal storage proteins include globulins which are soluble in saline solution and albumins which are water-soluble. In cereals, the globulin and albumin are concentrated in the aleurone layer, bran and germ and lower concentration can be found in the endosperm (Hoseney 1992). Cereal grains have relatively low protein content (10-12%) compared to legume seeds. In barley, the protein content varies between 7 to 14.6% (Guerrieri 2004). Wheat, barley and rye have similar amino acid composition (Guerrieri 2004). The storage

proteins, prolamins and glutelins are deficient of tryptophan, lysine and methionine. Threonine, after lysine is one of the limiting amino acid in barley. While the hull contains low protein concentration, its proteins are high in lysine. The germ proteins are high in lysine while the endosperm has lower lysine content but higher than in other cereals.

Prolamin is the major endosperm storage protein. Its name originates from the fact that it is rich in proline and amide nitrogen derived from glutamine (Shewry et al 2001). Prolamins in barley are referred to as hordein accounting for 40% of total proteins. However, prolamins are low in lysine. High contents of glutamic acid and proline have been found in the endosperm of barley (Hoseney 1992). Protein synthesis occurs throughout the fruiting season of the plant. Unlike proteins, starch is synthesized during fruiting and increases as the grain matures (MacGregor et al 1993).

#### ***1.3.1.4 Lipids***

Barley lipids make up approximately 3% of the kernel. The majority of the lipids are found in the germ. Other lipids are contained in the starch granules in quantities relative to amylose. Barley contains non-polar lipids (72%), glycolipids (10%) and phospholipids (21%) (Hoseney 1992). Barley fatty acids contain higher levels of linolenic acid and are more saturated than those found in wheat. A distinct characteristic of barley is that it contains all eight naturally occurring tocopherols and their identities were confirmed by gas-chromatography coupled with mass spectrometry (Govind et al 1972). The germ tissues contain tocopherols while the starchy endosperm and aleurone layer contain the tocotrienols.

### ***1.3.2 Minor Constituents***

#### ***1.3.2.1 Micronutrients***

Apart from being excellent sources of fibre, vitamins, and minerals (phosphorus, copper, manganese, selenium); barley also contains non-nutritive components that may provide substantial health benefits beyond basic nutrition. A study done by Ragaee et al (2006) comparing the composition of dietary fibre, resistant starch, total phenols and antioxidant properties of barley indicated that barley had the highest levels of phosphorous, calcium, potassium, magnesium, sodium, copper and zinc compared to millet, wheat, rye and sorghum. The aleurone layer contains the majority of the minerals (P, K, Mg, Ca, Zn and S) in cereals while the vitamins (Vitamin E, B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub>) are mainly found in the aleurone or scutellum.

### ***1.4 Phytochemicals***

Naturally occurring chemical substances in plants are referred to as phytochemicals (Zielinski et al 2000). Though a large percentage remains unknown, about 5000 of the phytochemicals present in plants have been identified (Adom et al 2002). These compounds exhibit antioxidant properties and help in the reduction of incidences of degenerative diseases. Experimental and epidemiological studies have shown the chemopreventive action of some phytochemicals (Kris-Etherton et al 2004). However, the evidence from epidemiological studies is on specific phenolics.

The most common phytochemicals are phenolic compounds. They belong to various groups of compounds and these include simple phenols, hydroxybenzoic and cinnamic acid derivatives, flavonoids and coumarines, among others (Naczki et al 2004). Research has shown that many phytochemicals found in fruits and vegetables are also detected in cereals. Genetics, environment and processing are the factors that influence the amount of phytochemicals in cereals (Zhou et al 2004; Mpofu et al 2006).

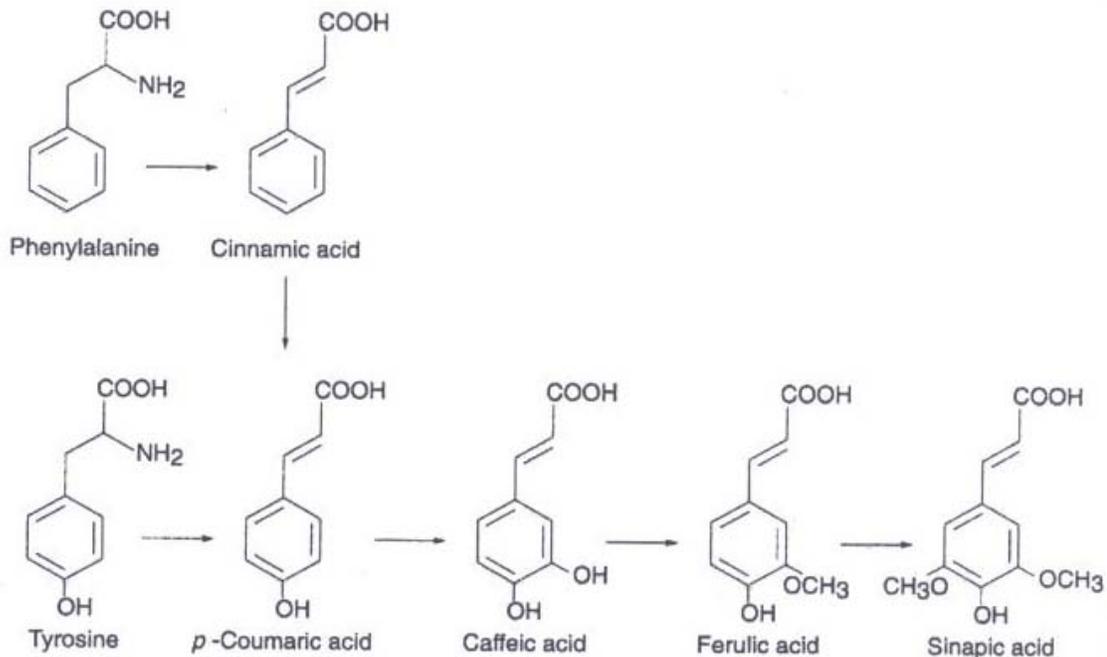
Several phenolic compounds are present in whole grains; however, bound phytochemicals are predominant. These phenolic compounds in plant-based foods exist as free or covalently bound to non-starch polysaccharides and account for the sensory characteristics such as the appearance, taste, smell and oxidative stability (Naczki et al 2004). The role of whole grain in the prevention of colon cancer is partly due to the fact that bound polyphenols may survive digestion and may reach the colon possessing anti-cancer effects. When bound phenolics reach the colon they are acted upon by microbial flora; consequently, the freed phenolics can now be absorbed by the colon endothelial cells (Sidhu et al 2007). The total dietary intake of phenolic compounds is estimated to be 1 g/day. It is 10 times higher than that of vitamin C and 100 times higher than vitamin E (Scalbert et al 2000).

#### ***1.4.1 Phenolic acids***

Phenolic acids are widely distributed in substantial quantities in cereals, fruits and vegetables. Cereal grains are a dietary source of phenolic acids which occur in free, conjugated and bound form. Studies have shown that the total amounts may reach approximately 500mg per

kg of edible cereals; however, flavonoids are present in small quantities in cereals (Zielinski et al 2000). Phenolic acids differ from phenols by their acidic character (Yu et al 2001).

Phenolic acids consist of two classes namely hydroxybenzoic acids and hydroxycinnamic acids. The former include gallic, *p*-hydroxybenzoic, vanillic, syringic and protocatechuic acids. The hydroxycinnamic acids include *p*-coumaric, caffeic, ferulic and sinapic acids. Hydroxycinnamates commonly found in barley grain (Von Wettstein 1985). A characteristic of the hydroxycinnamic acids is the C6-C3 structure (Harborne 2000). Phenolic acids are synthesized via the shikimate pathway (Figure 1.2) (Rice-Evans et al 1997). The hydroxycinnamates are formed through the hydroxylation and methylation of *p*-coumaric in position 3 and 5 (Shahidi et al 2004).



**Figure 1.2: Intermediates in the phenylpropanoid biosynthesis (Rice Evans et al 1999).**

Holtekjolen et al (2006) reported that antioxidant activity of grains depends on the structure of phenolic acids. The CH=CH-COOH group in the cinnamic acid derivatives gives higher antioxidant capacity compared to the COOH group of the benzoic acid derivatives. The radical scavenging activity of phenolic acids is highly dependent on their structure. For example, the number of hydroxyl groups on the benzene ring and the ortho substitution with electron donor methoxyl group both increase the stability of the phenoxyl radical (Rice-Evans et al 1996). Furthermore, *o*- or *p*- dihydroxylic groups that contain one free and one alkylated hydroxyl group (usually methoxyl) are stronger antioxidants. Based on structure, among the cinnamic acid derivatives, caffeic acid is a stronger antioxidant than ferulic acid, which is a more effective antioxidant, compared to *p*-coumaric acid (Holtekjolen et al 2006). Kikuzaki et al (2002) found that the radical scavenging activity using DPPH• was in the decreasing order caffeic > sinapic > ferulic > *p*-coumaric. A similar trend was also reported by Cuvelier et al (1992) when they evaluated the antioxidant capacity of the caffeic, sinapic, ferulic and *p*-coumaric in a methyl linoleate system. The introduction of a second hydroxyl group on the ortho or para position increases antioxidant activity hence caffeic acid is a more efficient antioxidant in vitro when compared to the monophenol, *p*-coumaric (Holtekjolen et al 2006).

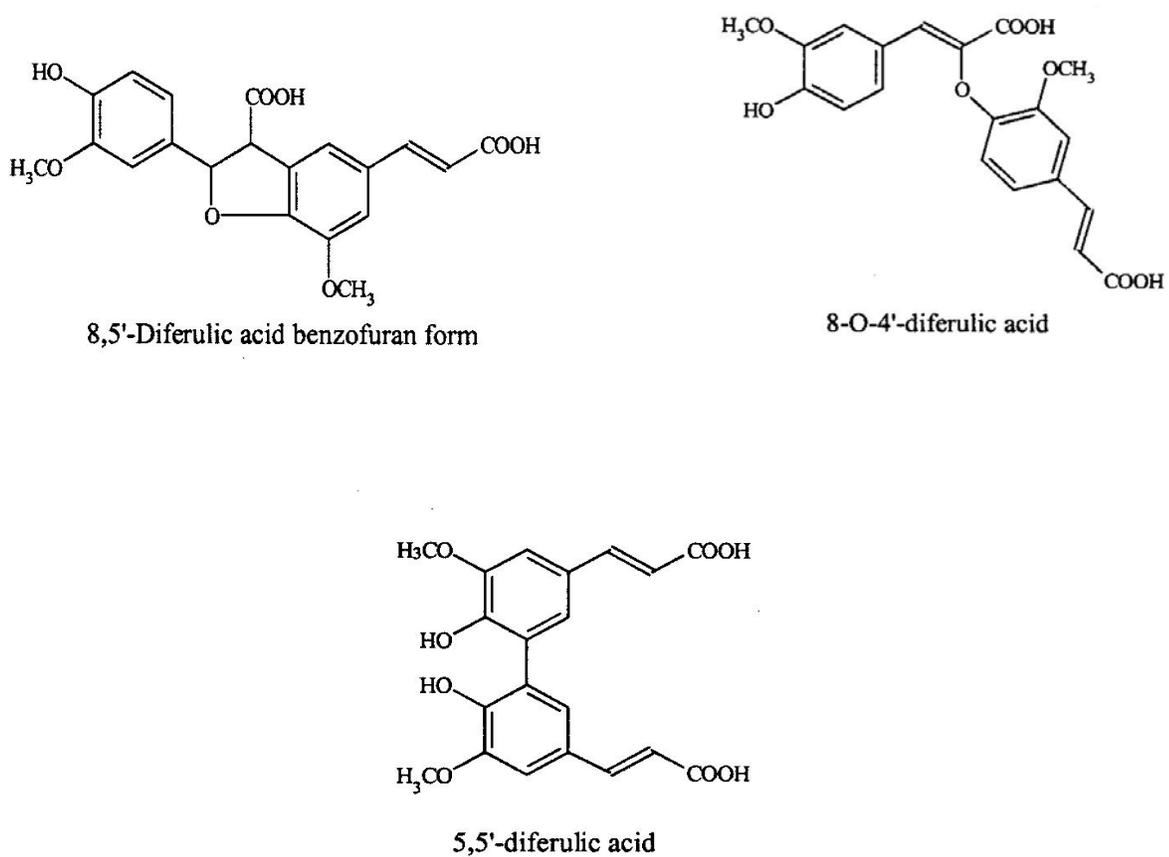
Ferulic acid (4-hydroxy-3-methoxycinnamic acid; FA) is the most abundant phenolic acid in cereals making approximately 90% of the total polyphenols (Nordkvist 1984, Yu et al 2001). This major low-molecular-weight phenolic acid is mainly found in the pericarp and aleurone cell layer esterified to the cell wall (Siebenhandl et al 2007). In a study done by Holtekjolen et al (2006), ferulic acid accounted for 52-69 % of the total amount of phenolic acid. In addition, the content of FA was related to type of grain with hulled varieties having significantly higher

content of FA in comparison to hullless. Phenolic acids such as ferulic acid and caffeic acid have been reported to possess antioxidant properties (Daniels et al 1967). Furthermore, ferulic acid can be used to predict end use of cereals (Pussayanawin 1986). The second most abundant phenolic acid is  $\rho$ -coumaric acid (Holtekjolen et al 2006, Hernanz et al 2001). Ferulic and  $\rho$ -coumaric are esterified to arabinoxylans and lignin (Bunzel et al 2004). Phenolic acids are distributed unevenly in the layers of the bran; consequently, different grain fractions have varying antioxidant activities. The outer layers contain the highest contents of phenolic acids while the starchy endosperm contains low levels (Holtekjolen et al 2006). Other phenolic acids such as protocatechuic, vanillic and chlorogenic have been identified and quantified in acid hydrolyzate of barley (Yu et al 2001).

In plant materials, the main ferulic acid dehydrodimers identified include 8-O-4'-DFA, 8-5' DFA benzofuran, 8-5' DFA and 5-5' DFA forms, with 8-O-4'-DFA being the most prominent (Andreasean et al 2000; Hernanz et al 2001; Qui et al 2009) (

**Figure 1.3).** Ferulic acid dehydrodimers from wheat bran have been reported to exert antioxidant activity in vitro (Garcia – Cornesa et al 1997). In barley grains, ferulic acid dehydrodimers have also been reported in varying contents (Hernanz et al 2001; Holtekjolek et al 2006). The formation of diferulic acid is facilitated by extracellular peroxidases through the formation of cross-links between cell wall constituents and phenolic acids (Biggs et al 1987). Ferulic acid dehydrodimers in different in vitro assays have also been reported to possess higher antioxidant properties than those of the monomer (Brett et al 1999; Waldron et al 1996). DFAs

vary depending on cereal type, varieties in one cereal and differences in dietary fibre fractions such as soluble or insoluble (Bily et al 2004; Bunzel et al 2001; Qiu 2009; Renger et al 2000).



**Figure 1.3: Structures of ferulic acid dehydrodimers in barley (Waldron et al 1996).**

Most quantitative studies specific to phenolic acids are performed by chromatographic analysis. Phenolic acids have been extracted in barley using hot water, acid, alkali and enzyme hydrolysis. Treatment with acid and alpha amylase released more phenolic acids (Yu et al 2001). Maillard et al (1995) also reported on the bound ferulic acid and  $\rho$ -coumaric acid in barley and malt using HPLC after alkaline hydrolysis. Using HPLC analysis of barley fractions, Madhujith et al (2006) identified phenolic acids similar to those in wheat (Li et al 2005; Li et al 2007). Losses of phenolic acids during base hydrolysis, acid hydrolysis or both have been reported in literature. In the determination of phenolic acid levels in rapeseed using 4 N NaOH for 4 hours under nitrogen, a loss of 10% was estimated for cinnamic acid derivatives such ferulic and  $p$ -coumaric acid. However, a loss of 67 and 37 % was reported for caffeic and sinapic acid respectively (Krygier et al 1982).

In order to map the location of ferulic acid (FA) in cereal grains, fluorescence techniques have been employed (Akin 1995, Fincher 1976, Fulcher et al 1972). The relative fluorescence assay was able to detect differences in cultivars in FA concentration in one study done by Zupfer et al (1998); however the measurement errors were large. Liberated bound phenolic acids have also been analyzed using HPLC-DAD-MS by Holtekjolen et al (2006) and the monomeric FA was the most abundant phenolic acid in barley flours from different varieties.

### ***1.5 Flavonoids***

A group of phytochemicals found in cereals are flavonoids which are located in the pericarp. These compounds are made up of C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub> skeleton that consists of two aromatic rings joined by three carbon link labeled A, B and C (Figure 1.4). The main classes of dietary

flavonoids include flavanols, flavones, flavanones, anthocyanidins, isoflavanoids and flavonols (Figure 1.5). Flavonoids are formed in plants from the aromatic amino acids phenylalanine and tyrosine and malonate (Neish 1986). Flavonoids have shown activity as scavengers of various oxidizing species including superoxide anion, hydroxyl radical and peroxy radicals (Harborne et al 2000).

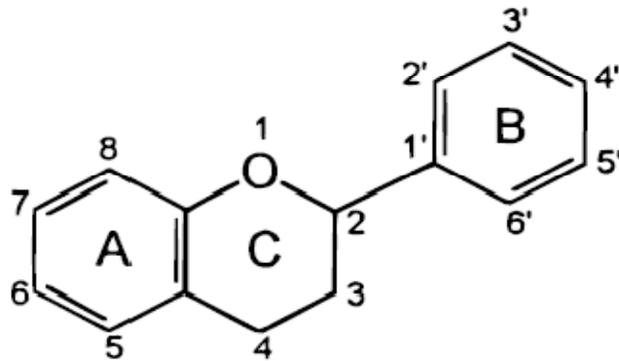
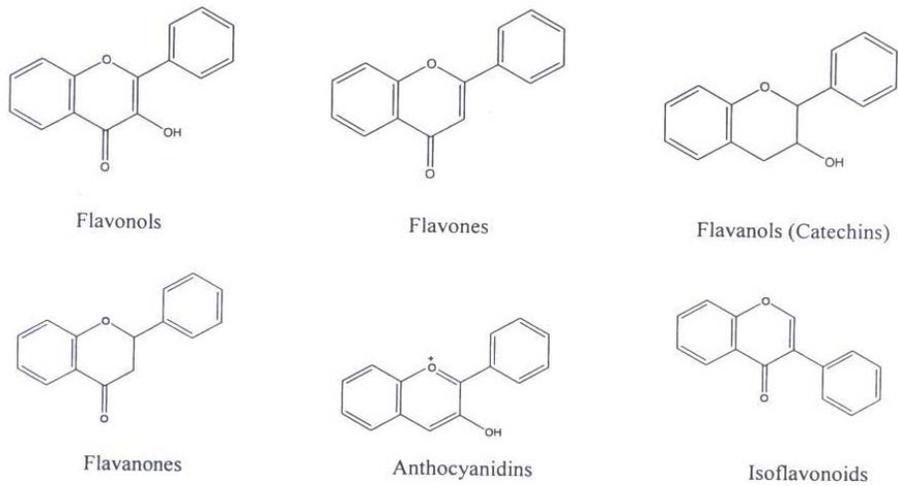


Figure 1.4: Basic flavonoid structure (Pietta 2000).



## **Figure 1.5. Structures of the main classes of dietary flavonoids**

Some of the physiological roles of flavonoids in plants as reviewed by Pietta (1999) include acting as visual signals for pollinating insects and acting as catalysts in light phased photosynthesis. They also regulate iron channels involved in phosphorylation and act as stress protectants in plant cells by scavenging reactive oxygen species (ROS) produced by the photosynthetic electron transport system. Flavonoids make up part of the defense system of plants as a result of their astringency. They also protect plants from UV radiation of the sun and scavenge UV- generated reactive oxygen species (Pietta 1999).

Flavonoids have health-promoting properties due to their high antioxidant capacity in both in vivo and in vitro systems (Cook et al 1996). Considerable attention has been directed towards flavonoids due to their beneficial effects as antioxidants in the prevention of human diseases such as cancer and vascular diseases and some pathological disorders of gastric and duodenal ulcers, allergies, vascular fragility and viral and bacterial infections (Zand et al 2002).

### ***1.5.1 Anthocyanins***

Anthocyanins are a major group of water soluble compounds that belongs to the flavonoid family found in pigmented cereals. There are over 250 naturally occurring anthocyanins which are *O*-glycosylated with different sugar substitutes including glucose, rhamnose, xylose, galactose, arabinose and fructose (Francis 1989). Anthocyanins are glycosides and acylglycosides of anthocyanidins. Colored grains have potential for use as food colorants and as functional food in whole grain products (Francis 1989; Hosseinian et al 2008).

Anthocyanins play a vital role in the mechanism of plant resistance to insect attack and also play the role of attracting animals in pollination and seed dispersal (Stark and Wary 1993).

The anthocyanin daily intake in humans in the US has been estimated to be as much as 180-215 mg /day (Kuhnau 1976). Some of the health functions associated with anthocyanins in human as reviewed by Lila (2004) include protection from DNA cleavage, altering development of hormone-dependent disease symptoms, enzyme inhibition, boosting the production of cytokines, lipid peroxidation, membrane strengthening and decreasing capillary permeability and fragility. Jing et al (2008) suggested that the structure of anthocyanin affected chemoprotection, which was measured as inhibition of colon cancer cell proliferation. Non-acylated anthocyanins had greater inhibitory effect on HT-29 cell proliferation than anthocyanins with pelargonidin, triglycoside and or acylation with cinnamic acid. Anthocyanin pigments and anthocyanin-rich foods have been reported to lower the risk of colon cancer through the inhibition of proliferation of human colon cancer in vitro (Zhao et al 2004). Role of anthocyanins in inhibiting colon cancer have been reported for in vivo studies (Jing et al 2008; Lala et al 2006). In addition, the inhibition of cancer cell growth through induction of apoptosis by anthocyanins has also been reported (Katsube et al 2003).

Cyanidin, delphinidin, malvinidin, pelargonidin, petunidin and peonidin are the common anthocyanins found in nature and have also been found in cereals. The stability of these compounds increases with the number of methoxyls in the B ring and decreases as hydroxyls increase. As a result, malvidin is the most common anthocyanidin followed by peonidin, petunidin, cyaniding and delphinidin (Timberlake et al 1988). Pigment distribution varies

throughout the kernel. The blue pigments in wheat are located in the aleurone layer while the purple pigments are found in the pericarp layers (Abdel-Aal et al 1999). A study by Moreno et al (2005) observed that the anthocyanins in corn were concentrated in the pericarp. Due to the presence of anthocyanins and melanins, barley exists in colors black, blue, red and purple (Takahashi 1955). In barley, anthocyanins are found in the pericarp or in the aleurone layer causing the kernel color to be blue. Some barley varieties (*Hordeum vulgare*) have a black pigmentation due to a melanin-like pigment which may overlap purple or blue color as a result of anthocyanins (Siebenhandl et al 2007).

Traditionally, paper and / or thin layer chromatography, as well as UV-Vis spectroscopy have been used in the identification of anthocyanins. Cyanidin 3-glucoside and peonidin 3-glucoside were identified as the most abundant anthocyanins in purple wheat and rye using paper chromatography (Deido et al 1972). Analytical techniques such as capillary zone electrophoresis (Ichikawa et al 2001) and combination of chromatography and electrophoresis are becoming popular. However, LC has been employed to separate, identify and quantify these anthocyanins in cereals. This standard method can also be used for both preparative and analytical purposes.

Total anthocyanin contents of two- and six-rowed black barley were reported (Siebenhandal et al 2007). Fractionation of the grains exhibited an effect on the anthocyanin content and thus the antioxidant properties. The bran contained the highest levels of anthocyanins. The anthocyanins are located in the lemma and pericarp making part of the bran fraction. The anthocyanin content in 127 lines of colored barley varieties using HPLC was evaluated (Kim et al 2007). Anthocyanin composition of colored cereal grains was also studied

by Abdel-Aal et al (2006). The separation, identification and quantification were achieved using liquid chromatography (LC). Extraction of anthocyanins can be achieved using acidified organic solvents such as methanol and acetone (Shahidi et al 2000).

Acidified methanol has shown the highest extraction efficiency (Mazza et al 2004; Naczka et al 2006). These solvents have the ability to destroy plant cell membranes and dissolve the anthocyanins. In the analysis of phenolics using spectrophotometric techniques, there is usually overestimation due to interferences by other UV- absorbing compounds such as proteins, nucleic acids and amino acids. Effect of temperature on the separation of anthocyanins has also been evaluated by Abdel-Aal et al (2006). To increase column efficiency whilst maintaining column quality and preventing degradation of anthocyanins the optimum temperature employed was 50°C for purple corn, pink corn and purple wheat (Abdel-Aal et al 2006).

Liquid chromatography coupled with mass spectroscopy and nuclear magnetic resonance (NMR) have been reported to be the most powerful techniques in revelation of structural profiles of anthocyanins. Currently the most satisfactory method for mixture analysis involves a multistep method of separation, isolation and quantification by LC coupled with MS and high-field NMR for peak identification (Mazza et al 2004).

### ***1.5.2 Flavanols***

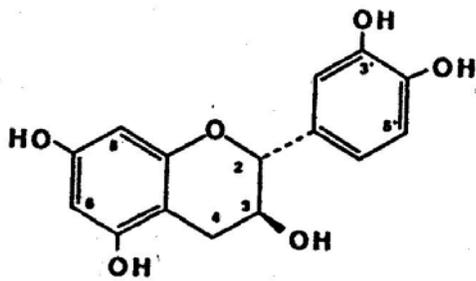
One sub-class of flavonoids are the flavan-3-ols which consists of catechin and catechin gallates. Proanthocyanidins (condensed tannins) are oligomeric and polymeric flavan-3-ols that produce anthocyanidins by cleavage of a C-C interflavanyl bond under strongly acidic conditions (Sun et al 1998).

Flavanols are the major free phenolics in barley (Dvorakova et al 2008). Proanthocyanidins (PAs) are mixtures of dimers and higher oligomers of monomeric flavan-3-ol linked mainly through the C4-C8 and to a lesser extent through C4-C6 linkage (Harborne 2000). They are also referred to as condensed tannins or procyanidins. The monomer forms include (+)-catechin and (-)-epicatechin and the polymer forms consists of units of (+) - catechin and (+)-gallocatechin. A study by Meridith et al (1985) also showed that proanthocyanidins may influence dormancy and germination.

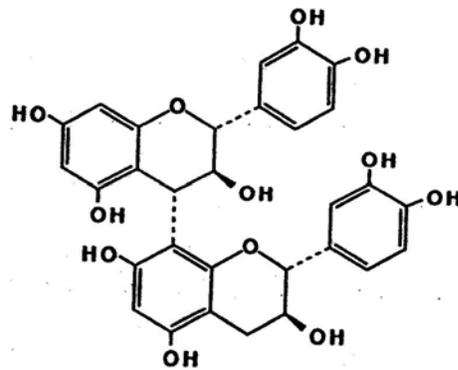
Oligomeric and polymeric flavan-3-ols play a role in the determination of nutritional quality and organoleptic properties of food (Scalbert 1992). Some animal trials have shown the antinutrient effects of proanthocyanidins. Furthermore a negative correlation between flavanol content and protein digestibility has been reported (MacGregor et al 1993). In contrast, some health benefits of flavanols have been studied (Hollman et al 1996; Yao et al 2004).

Proanthocyanidins in barley bran have shown similar or higher antioxidant activity in comparison to catechin (Tamagawa 1999). There are also known to have antioxidant activity and health benefits (Kris-Etherton et al 2004). Barley contains PAs which are monomers, dimers, trimers and polymers unlike in sorghum where the proanthocyanidins are polymers (Awika et al 2003; Gu et al 2002; Gu et al 2004; Holtekjolen et al 2006). Compared to monomeric phenolics, PAs have higher antioxidant capacity in vitro (Hagerman et al 1998). In barley the PA oligomers that have been detected include the two dimers namely procyanidins B3 and prodelfphinidin B3 (Figure 1.6) and the four trimers namely procyanidin C2, prodelfphinidin C2 and two other prodelfphinidins (Friedrich et al 2002, McMurrough et al 1996). Holtekjolen et al (2006)

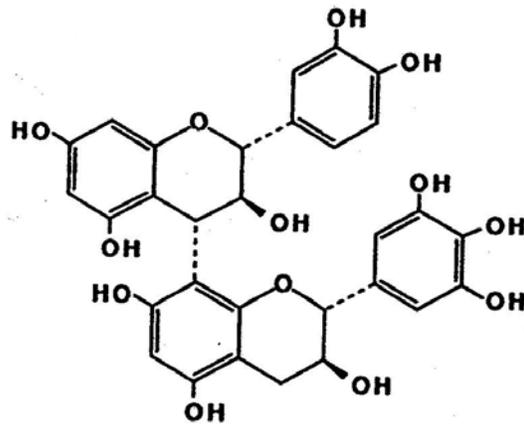
analyzed the flavanol content in different barley varieties and the results showed that the major flavanols were the trimers making up 53-61% of the total flavanols.



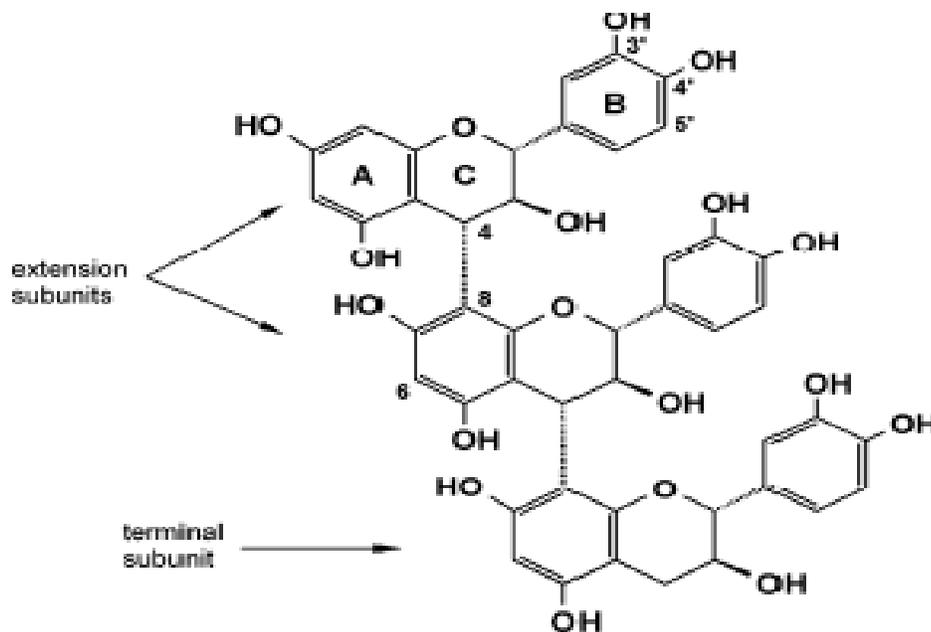
(+)-Catechin



Procyanidin B3



Prodelphinidin B3

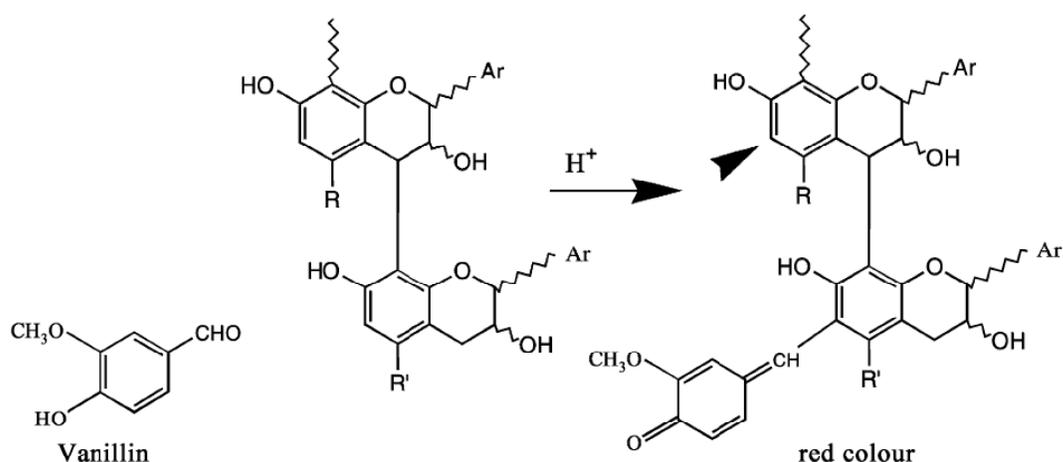


**Figure 1.6: Structures of flavan-3-ol monomeric, dimeric units and representative structure of a (+)-catechin based trimeric procyanidins (McCallum et al 1990).**

Flavanol glycosides are naturally occurring in plants and appear as 3-, 5-, 7- *O* glycosides and as 6- and 8-*C* glucosides (Harborne et al 1999). The catechin glucoside has been reported by Dvorakova et al (2008) in 10 barley varieties. Friedrich et al (2002) isolated the catechin glucoside from 20 barley varieties.

Methods of quantifying flavanols vary from simple non selective colorimetric assays to more sophisticated HPLC methods. Quantification of PAs can be achieved by use of the vanillin-HCl assay. The vanillin-HCl assay involves the reaction between an aromatic aldehyde vanillin with a meta substituted ring of flavanols to yield a red adduct (Figure 1.7). However, not many researchers use the vanillin-HCl assay for quantification of PAs because an appropriately

substituted flavanol which is not a condensed tannin is likely to react with vanillin (Sun et al 1998). Critical factors in this assay include type of solvents used, the nature and concentration of acid, reaction time, vanillin concentration and the type of reference standard used (Scalbert et al 1992; Sun et al 1998). PAs in colored barley have been evaluated by Kim et al (2007) and Dzorakova et al (2008) using the vanillin assay (Sun et al 1998).



**Figure 1.7: Chemistry of the vanillin-HCl assay for the condensed tannins. The arrowhead points to a second potentially reactive site (Schofield et al 2001).**

Other techniques that have been employed in the quantification of proanthocyanidins in food include HPLC-DAD-EIMS (Tomas-Barberan et al 2001), reversed phase HPLC and post-column derivatization (de Pascual-Teresa et al 2000), protein precipitation assay (Price et al, 1980). The fragmentation pathways for PAs are can be achieved through heterocyclic ring fission (HRF)  $[M-C_6H_6O_3-H]$ , interflavanic bond cleavage through quinone-methine (QM) mechanism and through the retro-Diels-Alder (RDA) cleavage, and subsequent water elimination as shown (**Error! Reference source not found.**) (Verardo et al 2008). The detection of PAs in malt was

successfully achieved by use of HPLC in combination with electrospray ionization mass spectrometric detection in the negative mode (Friedrich et al 2000).

**Error! Reference source not found.: ESI mass spectrum of prodelphinidin B3 (full MS) and mass spectra achieved by multiple stage trap collision induced dissociation (CID) (MS<sup>n</sup>) and source CID (Friedrich et al 2000).**

### ***1.6 Barley Food Uses***

Barley has many important food uses worldwide. In Korea, pearled barley is used as a rice substitute and as an ingredient in the manufacturing of soy paste and sauce (Ryu 1979). Roasted barley is also used as a tea or coffee substitute. Many countries include barley in their traditional dishes such as *kasha* in Russia and Poland, *tsampa* in Tibet, *miso* in Japan and *sattu* in India (Macgregor et al 1993). In food products, a small proportion of barley malt is used for flavor and color enhancement while the majority of barley malt is utilized in the production of alcoholic beverages. Pearled barley can be utilized in soups and may also be reduced to flour incorporated in breakfast cereals, stews, bakery flour blends, porridge and or baby foods (Baik et al 2008).

Barley flour has been incorporated into wheat-based products to make cakes, bread, cookies and noodles. A study by Trogh et al (2005) reported that bread baked from wheat-barley flour blend had similar loaf volume and maintained crumb softness for a longer period of time in comparison to bread baked from wheat flour alone. Furthermore, the bread had higher content of soluble dietary fibre. Izydorczyk and colleagues (2005) studied the effect of incorporating waxy and high amylose hullless barley in white-salted and alkaline noodles. The results showed an

increase in water absorption, firmness and resistance to compression of the noodles. Reduction in cooking time and a darkened color of dough was also observed.

## ***1.7 Processing Barley Grain***

Grain processing involves the partial or complete removal of the bran and this results in loss of micronutrients and phytochemicals which are related to health benefits attributed to the consumption of grains and grain products. Most barley varieties are covered with a tough inedible hull, though now some genetically hullless varieties are available. This hull must be removed in order for the kernel inside to be used for human food. Dehulling involves the removal of the hull and small portions of the bran, germ and endosperm.

### ***1.7.1 Milling***

Milling involves the removal of the pericarp, seed coat, nucellar epidermis, aleurone layer and germ. This is done so as improve palatability and increase shelf-life. This process can be achieved by use of a hammer mill. Decortication of grain to produce bran results in the concentration of the phenols. The bran can then be incorporated into food products such as bread, cookies and tortilla enriching them with dietary fibre and nutraceuticals (Dykes et al 2007). An important primary process in food barley utilization is pearling which involves the removal of grain tissues through abrasive action starting from the outer layer of grain (Bellido and Beta 2009; Yeung & Vasanthan 2001). Pearling a hullless or dehulled barley grain further removes the remaining bran, germ and part of the endosperm leaving a central endosperm rich in starch,  $\beta$ -glucans, hordeins and glutelins. The more the barley is pearled, the lighter in color the end product appears. Pearled barley can be processed into grits, flakes and flour. “Cut barley”

can be produced by cutting pearled barley along the crease and further polishing. This makes the grains resemble polished rice grains in terms of size and shape. The cutting of barley reduces the cooking time through increased water imbibitions. Therefore, barley can be used as a substitute for rice (Baik et al 2008). Infrared heat treatment when applied to barley in food products also reduces the cooking time, improves the nutritional quality (fibre content), extends shelf-life, modifies grain structure and helps maintain the color of end-product (Ames et al 2006).

### ***1.7.2 Malting***

Apart from being traditionally acceptable, barley is ideal for malting because its hulls protect the malted grain and function as filter aids in the subsequent brewing steps (Hoseney 1992). In the early stages of brewing process, barley malt contributes to the phenolic and polyphenolic content of beer (Shahidi et al 1995). In beer, 80% of the phenolics originate from barley malt. These compounds contribute to the sensory properties of beer and participate in chill haze formation and overall stability of beer. Goupy et al (1999) analyzed the antioxidant composition and activity of barley, malt extracts and isolated phenolic compounds. The analysis showed that the phenolic content decreased after malting. Apart from polyphenols, both the barley and malt extracts also contained other phytochemicals including carotenoids (lutein and zeaxanthin) and tocopherols ( $\alpha$ ,  $\delta$  and  $\gamma$ ). During lactic acid fermentation of barley bran, a purple pigment called hordeumin, a type of anthocyanin/tannin pigment was produced. The antioxidant activity of hordeumin increased with the increase in fermentation time (Deguchi et al 2000).

### ***1.7.3 Sprouting***

Sprouts are common ingredients of vegetable salads and are often eaten raw. Compared to cereal grains and their products, cereal sprouts are believed to have a greater nutritive value (Price 1988). Sprouting of cereals has been reported to alter the taste and texture of food (Finney 1978). During sprouting, modification of the barley grain begins at the proximal end of the kernel, adjacent to the scutellum (

**Figure 1.1).** Some of the enzymes that are activated by the germination process include endo-(1→3), (1→4) β-glucanase, endo-(1→3) - β-glucanase, endo-(1→4) β-xylanase, endopeptidase, carboxypeptidase, α-amylase (types I, II, III), β-amylase, α-glucosidase, lipase, phospholipase and limit dextrin (Newman et al 2008). The enzymes are synthesized in the aleurone tissue and migrate into the endosperm to effect hydrolysis of the cell walls, proteins and starch (Macleod et al 1964). Diastatic activity, a measure of the activity of hydrolytic enzymes was found to increase steadily when kidney beans were sprouted (Mwikya et al 2001). Addition of moisture and control of temperature (14-18°C) activates enzyme systems and hormones for sprouting (Newman et al 2008). To enhance and improve germination, hypochlorite, calcium hydroxide, sodium hydroxide, or sodium carbonate can be added.

Cruciferous sprouts are also associated with inducing carcinogen-detoxifying enzymes systems (Fahey et al 1997). Dietary in vitro and human intervention studies have indicated that cruciferous and leguminous sprouts can decrease H<sub>2</sub>O<sub>2</sub> - induced DNA damage which in turn may lower the risk of certain cancers (Gill et al 2004).

Traditionally sprouting was used to remove anti-nutritional factors in legumes. Trypsin inhibitor activity, phytates and tannin content have been found to decrease with sprouting

(Khokhar et al 1986, Mwikya et al 2001). However, sprouting has been applied to legumes to improve the nutritional quality (Khokhar et al 1986; Wu et al 1953). Changes in nutritional content are accompanied by dry matter losses (Chung et al 1989; Mwikya et al 1999). Loss in dry matter during sprouting is due to degradation and oxidation of starch and sugars during respiration for the provision of energy for the increased metabolic functions in the sprouting seed. During the respiration, carbon dioxide and water are released and escape from the seed. Some of the changes in nutritional composition reported include increase in ascorbic acid content in soya beans and wheat grain (Wu et al 1953; Yang et al 2001), decreased starch content in finger millet (Mwikya et al 1999), increased protein digestibility, crude fibre and diglyceride in barley (Chung et al 1989).

Sprouting has been applied to cereals such as rice, corn and wheat. Barley has been sprouted hydroponically (Chung et al 1989). When applied to cereals and legumes, sprouting can improve the palatability, nutritional quality and acceptability of food products. Germinated cereals and legumes have been employed in weaning food formulation. Sprouting has been applied to cereals and legumes in order to decrease the dietary bulk and increase the nutrient density (Marero et al 1988).

Antioxidant capacity of commonly consumed sprouts such as alfalfa, broccoli and radish has been investigated. Young sprouts have been found to be richer in phenolic compounds than older sprouts. Some of the phenolic compounds that have been identified in alfalfa sprouts include p-coumaric, ferulic acid, myricetin and luteolin-7-*O*-glucoside. Sinapic acid was identified in broccoli sprouts while rutin was identified in radish (Oh and Rajashekar 2009). The

higher phenolic content exhibited by the young sprouts indicated that there were likely to provide more health benefits. Total phenolic content in broccoli sprouts has been reported to decrease with age of plant (Oh and Rajashekar 2009). Mung and fava bean sprouts have been found to contain concentrated phenolics (Randhir et al 2004). Sprouting was also found to increase the concentration of phenolic acids in wheat grain (Yang et al 2001). There are not many reports on effect of sprouting on the phenolic composition of barley grains. Most of the literature on sprouting is centered on nutritional profiles. Knowledge on variation in different barley genotypes is therefore scarce. Apart from being a health food, seed sprouts also have phytochemicals with health promoting properties (Randhir et al 2004).

## **1.8 Antioxidant Assays**

### **1.8.1 Biological assays**

Phenolic compounds found in barley exhibit antioxidant activities. Antioxidant capacity can be quantified by the use of several chemical and biological techniques based on either current state of oxidation or the radical scavenging ability. An ideal *in vitro* chemical antioxidant assay should be quick with simple quantification of antioxidant capacity (Sharma et al 2009). An integral part of the human metabolism is the production of reactive oxygen species (ROS) including free radicals. The ROS, reactive nitrogen species (RNS) and free radicals have potential damage to biological systems. Cells and biological fluids have antioxidant systems that prevent free radical formation and repair oxidative damage (Halliwell 1996).

Use of *in vivo* techniques to confirm antioxidant status after consumption of dietary antioxidants is crucial. The *in vitro* assays provide information regarding to the antioxidant

potential of the antioxidants *in vivo*. The main ROS that cause oxidative stress in the body include superoxide anion, hydrogen peroxide, peroxy radical, hydroxyl radical, singlet oxygen and peroxynitrite (Sánchez-Moreno 2002). Peroxidase based systems are used to measure the peroxide scavenging activity. The hydroxyl radical scavenging activity is calculated using “deoxyribose assay” which measures the inhibition of the degradation of deoxyribose (Halliwell 1990). Peroxynitrite scavenging activity can be measured with a microplate fluorescent spectrophotometer in the presence of an antioxidant (Chung et al 1998).

### ***1.8.2 Chemical Antioxidant Assays***

Chemical antioxidant assays can be applied to food and biological systems. The chemical antioxidant assays can be divided into two groups namely electron transfer (ET) methods and hydrogen transfer methods (HAT). The electron transfer method is characterized by the change in color during the reduction of an oxidant (Huang et al 2005). The latter involves competition between the antioxidant and substrate (probe) for free radicals (Huang et al 2005). Examples of electron transfer assays include trolox equivalent antioxidant capacity (TEAC), the ferric reducing ability of plasma (FRAP) assay, copper reduction assay (CUPRAC) and the 2, 2-diphenyl-picrylhydrazyl radical scavenging activity (DPPH) assay. The hydrogen atom transfer assays include the crocin bleaching assay, the total peroxy radical trapping antioxidant parameter (TRAP) assay, and the oxygen radical absorbance capacity (ORAC) assay. However, these chemical assays can be applied to biological samples. Other antioxidant assays include total scavenging capacity (TOSC), chemiluminescence, electrochemiluminescence and the inhibition of Briggs-Rauscher oscillation reaction (Huang et al 2005), while other methods are still being developed. Radical scavenging assays such as ORAC (Bellido & Beta et al 2009),

DPPH (Beta et al 2007), ABTS (Zielinski et al 2000), ferric reducing antioxidant power (FRAP) (Siebenhandl et al 2007), total radical trapping antioxidant parameter (TRAP), photochemiluminescence (PCL) (Beta et al 2007) and superoxide radical scavenging assay (Madhujith et al 2006) have been employed in the determination of antioxidant activity in barley.

A study by Stratil et al (2007) on chemical antioxidant assays revealed that the antioxidant capacity values obtained from various experiments depend on the method used including reactivity of the radical, standards applied, and the reactivity of the compounds in the extracts. Tabart et al (2009) also reported that DPPH was the least sensitive when they evaluated the antioxidant capacity of various beverages using trolox equivalent antioxidant capacity (TEAC), ORAC, DPPH, superoxide scavenging capacity assay and haemolysis (red blood cell resistance to oxidative stress). According to the study by Tabart et al (2009), phenolic acids had the same DPPH radical scavenging activity as Trolox; however, cyanidin-3-glucoside and catechin had lower antioxidant activity compared to Trolox.

#### ***1.8.2.1 DPPH radical scavenging assay***

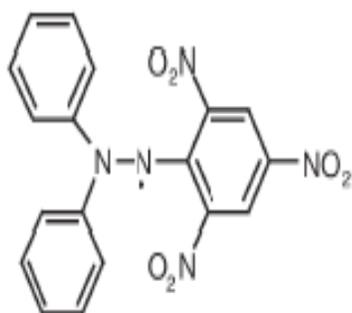
The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method is one of the oldest and frequently employed to assess the antioxidant capacity or potential of food extracts in a hydrophilic medium. It is one of the short methods that can be used to establish the hydrogen donating potency of compounds (Blois 1958). DPPH is a stable organic nitrogen radical that has a deep purple color (**Figure 1.8**). Due to its stability and differences in reactivity with peroxy radicals, many antioxidants that react quickly with peroxy radicals in vivo may react slowly or even be inert to DPPH (Huang et al 2005).

Various researchers have used the DPPH radical scavenging assay as a tool to quantify the antioxidant capacity using different protocols thereby making the comparison of results difficult. These protocols differ in the concentration of DPPH, temperature, incubation time, reaction, reaction solvents and the pH of the reaction mixture. Absorbance profiles of DPPH were evaluated in buffered methanol and ethanol and the results elucidated that the higher absorbance which was found in the buffered methanol solution had implications on the sensitivity (Sharma et al 2009).

The increase in concentration of the DPPH in the reaction may give absorbance readings beyond the accuracy of spectrophotometers (Ayres 1949). Sharma et al (2009) found that a concentration between 25-70  $\mu\text{M}$  DPPH and the use of buffered methanol or methanol was the most suitable to assess the antioxidant activity of non polar and polar compounds. Most of the phytochemicals are light sensitive hence all the operations are to be conducted in the dark/dim light since some of the phenolic compounds are prone to photodecomposition. A good DPPH assay should take into account spectrophotometric sensitivity range, sensitivity of DPPH to light, pH and solubility of the compound.

The DPPH radical scavenging assay has been employed in determining the antioxidant capacity of pearled wheat fractions (Beta et al 2005), barley flour (Bonoli et al 2004), barley hullless varieties (Beta et al 2007), pearled barley fractions (Madhujith et al 2006), Chinese black grained wheat (Li et al 2005), whole grain cereals (Ragee et al 2006) and colored barley varieties (Kim et al 2007). Unhulled barley (67%) has been reported to have higher radical scavenging capacity compared to hulled barley (64%) (Kim et al 2007). In the study by Beta et al (2007) the

DPPH radical scavenging capacities of barley genotypes varied from 13 to 27%. The variation in antioxidant properties in grains is partly attributed to the genetic and environmental variation (Mpofu et al 2006). Quantification of antioxidant activity in barley using the DPPH method has been reported (Zhao et al 2006, Kim et al 2007). Tabart et al (2009) reported that DPPH was the least sensitive when they evaluated the antioxidant capacity of various beverages using Trolox equivalent antioxidant capacity (TEAC), ORAC, DPPH, electron spin resonance (ESR) and haemolysis.



**Figure 1.8: Structure of DPPH free radical (Huang et al 2005).**

### **1.8.2.2 Total Phenolic Content assay**

The Folin-Ciocalteu method also referred to as the total phenols assay, involves a redox reaction between the phenolate ion produced by the dissociation of the phenolic proton and folin reagent. This assay involves the dissociation of phenolic proton which leads to the formation of a phenolate ion which has the capacity to reduce the Folin-Ciocalteu reagent. Basically, the Folin-Ciocalteu method measures the reducing potential of an extract. Its chemical nature is not clear but is believed to contain heteropolyphosphotungstates-molybdates (Huang et al 2005). The Folin-Ciocalteu reagent measures other constituents such as proteins apart from phenolics

thereby making its specificity poor. Shahidi and Naczk (1995) reported that this reagent detects all the phenolic groups found in the extracts including those found in extractable proteins.

The Folin-Ciocalteu method measures the total phenolic compounds expressed as ferulic (Mpofu et al 2006) or gallic acid equivalents (Zhao et al 2008, Dvorakova et al 2008, Zhao et al 2006). The original total phenolic content (TPC) assay by Singleton et al (1965) used gallic acid as a standard, however, other standards such as catechin, tannic acid, chlorogenic acid and caffeic acid have also been employed (Karadag et al 2009). The use of gallic acid as standard for TPC in barley is likely to result in low estimation. The absorbance values obtained for the TPC assay depend on number of hydroxyl groups and molecular structure of phenolic compound (Stratil et al 2006). In a study by Stratil et al (2006), the absorbance values for caffeic acid (two reacting OH) is approximately twice and for catechin (three reacting OH) three times higher than that of phenol (one reacting OH). Gallic acid had a higher absorbance than ferulic acid (Stratil et al 2006). There is a high likelihood that a highly reactive standard will give high absorbance and this will result in low values being measured in the samples.

### ***1.8.2.3 Oxygen Radical Absorbance Capacity (ORAC)***

The ORAC assay involves a competition reaction between antioxidants (phenolic compounds) and probe (substrate) for the peroxy radical generated by the azo compound, AAPH. During the ORAC assay free peroxy radicals are constantly being generated by 2,2'-azobis (2-methyl propionamide) dichloride (AAPH). The ORAC assay has been widely used to measure antioxidant activity of botanical and biological samples (Cao et al 1998). The assay is useful for food samples that contain multiple ingredients and have complex reaction kinetics

(Huang et al 2005). It can also be used with different free radical generators since the antioxidant capacity of compounds varies depending on the free radical employed in the assay (Karadag et al 2009). Accurate total ORAC values can be obtained by measuring the lipophilic and hydrophilic fractions.

The first version of ORAC employed made use of B-phycoerythrin (a fluorescent protein) (B-PE) as a probe (Cao et al 1993) but suffered many shortcomings. Ou et al (2001) substituted B-PE with fluorescein (FL), a synthetic non-protein probe. This unique method results in a complete reaction with a combination of inhibition time and level of inhibition (Tabart et al 2009).

In the ORAC study conducted by Tabart et al (2009), the antioxidant activities of anthocyanins, flavan-3-ols and phenolic acids were respectively 4-6, 3-8, 3-5 times higher than that of trolox using the peroxy radical. Results from these studies clearly elucidate that the various phenolic compounds (flavonoids, phenolic acids) react very differently with the free radicals such as peroxy and DPPH employed in the antioxidant assays. Antioxidant potential of pearled barley fraction was assessed using this technique (Madhujith et al 2006). Wang et al (1997) determined the antioxidant capacity of 14 anthocyanins including their aglycons using ORAC assay and cyanidin-3-glucoside had the highest ORAC activity which was 3.5 times stronger than trolox.

Based on the various antioxidant studies conducted, there has been a consensus that no single assay is adequate for evaluating the antioxidant capacity of foods due to the widely variable results that have been obtained. Furthermore, an individual antioxidant assay simply

reflects the chemical reactivity of the sample under specified conditions specific to that assay and consequently can be misleading when making conclusions.

## **Chapter 2.      *Proanthocyanidins, Anthocyanins and Phenolic Acids in Nine Food Barleys of Diverse Origin***

### **2.1   *Abstract***

Grains contribute phytochemicals that complement those found in fruits and vegetables. These phytochemicals, though minor compounds, contribute to the antioxidant properties which are related to the health benefits associated with the consumption of whole grain. Nine barley genotypes of diverse origin namely CI2230 from Nepal, CI1248 from Israel, 3 Peruvian genotypes; Peru 3, Peru 16 and Peru 35, Hokuto Hadaka from Japan, EX116; a cross between Moroccan and Canadian genotype, EX83; a cross between two Canadian genotypes and EX127; a cross between Canadian and German genotypes were studied. The genotypes were categorized based on appearance into purple, black and yellow grains. Phenolic compounds including phenolic acids and flavonoids were identified and quantified in these diverse genotypes using HPLC-ESI-MS analysis. The main classes of dietary flavonoids studied in the barleys were anthocyanins and flavan-3-ols. Phenolic acids that were identified and quantified included ferulic (246-485  $\mu\text{g/g}$ ) which was the dominant phenolic acid, sinapic (5-35 $\mu\text{g/g}$ ), caffeic (4-15  $\mu\text{g/g}$ ), vanillic (6-11  $\mu\text{g/g}$ ) and *p*-coumaric (5-90  $\mu\text{g/g}$ ). EX127 and Peru 35 had the highest levels of

total phenolic acids. Three ferulic acid dehydrodimers (8-0-4' DFA, 8-5' benzofuran form and 5-5' DFA) were also identified. Peru 35 had the highest contents of the most abundant DFA (8-0-4' DFA). The contents of 8-0-4' DFA and 8-5' benzofuran form ranged from 13 to 34  $\mu\text{g/g}$  and 10 to 25  $\mu\text{g/g}$  respectively. Using a spectrophotometric assay, the total anthocyanin content (TAC) varied significantly among barley genotypes, ranging from 8 to 38  $\mu\text{g/g}$ . CI1248, Peru 16, CI2230 and EX127 were the dark colored genotypes that exhibited relatively high TAC. The most abundant dimeric flavan-3-ols were procyanidins B3 (27-116  $\mu\text{g CE /g}$ ) and prodelphinidin B3 (40-130  $\mu\text{g CE /g}$ ). CI2230 (1029  $\mu\text{g/g}$ ) had the highest total flavonoid content and exhibited high antioxidant activity in the acetone extract. The monomeric unit, (+)-catechin (7-45  $\mu\text{g CE /g}$ ), was the most abundant while catechin glucoside ( $m/z$  451) was also identified. Among the Peruvian genotypes, Peru 16 and Peru 35 exhibited relatively high levels of total PA content. The total amount of flavonoids ranged from 441 to 1029  $\mu\text{g CE /g}$  using the vanillin assay. These barley genotypes were found to contain different quantities of phytochemicals and had high proanthocyanidin content thereby rendering them as alternative sources of antioxidants.

## **2.2 Introduction**

Barley (*Hordeum Vulgare* L.) is an important crop for direct human consumption, animal feed and industrial applications. Apart from being used for malting and as feed, other uses of barley include breakfast cereals, stews, soups, bakery flour blends, porridge and or baby foods as reviewed by Baik et al (2008). The health benefits associated with barley grain may be attributed to the presence of phytochemicals and their antioxidant properties.

A major source of dietary phenolics are phenolics acids which can be categorized into hydroxybenzoic and hydroxycinnamic acid derivatives. The latter includes coumaric, caffeic, ferulic and sinapic acids and these are commonly found in barley grain (Von Wettstein 1985). Whole grains are a main source of phenolic acids especially ferulic acid (4-hydroxy-3-methoxycinnamic acid) which has been found to be the most abundant phenolic acid in barley (Nordkvist 1984) and in other cereals such as wheat (Mpofu et al 2006). The formation of diferulic acid is facilitated by extracellular peroxidases through the formation of cross-links between cell wall constituents and phenolics acids (Biggs et al 1987).

Flavonoids exist as monomers and polymers. Flavan-3-ols are the major free phenolics in barley (Dvořáková et al 2008). The monomer forms include (+)-catechin and (-)-epicatechin and the polymer forms consists of units of (+)-catechin and (+)-gallocatechin. Proanthocyanidins are mixtures of dimers and higher oligomers of monomeric flavan-3-ol linked mainly through the C4-C8 and, to a lesser extent through C4-C6 linkage (Harborne 2000). They are also referred to as condensed tannins or procyanidins. Compared to monomeric phenolics, condensed tannins have higher antioxidant capacity in vitro (Hagerman et al 1998).

Pigmented grains have potential for use as food colorants and as functional food in whole grain products. Anthocyanins are a major group of compounds found in pigmented cereals and are water soluble. They have been reported to have higher antioxidant activity compared to vitamin C and E (Rivas-Gonzalo et al 2003). Due to the presence of anthocyanins and melanins,

barley exists in colours black, blue, red and purple (Takahashi 1955). In barley, anthocyanins are found in the pericarp or in the aleurone layer causing the kernel colour to be blue.

Antioxidant capacity can be quantified by the use of several chemical and biological techniques based on either current state of oxidation or the radical scavenging ability. The chemical antioxidant assays can be divided into two groups namely electron transfer (ET) methods and hydrogen transfer methods (HAT). The electron transfer method is characterized by the change in color during the reduction of an oxidant while the latter involves competition between the antioxidant and substrate (probe) for free radicals (Huang et al 2005).

Several aqueous solutions have been employed in the extraction of free phenolic compounds from barley including methanol, acetone, water and ethanol and their combinations (Nazck et al 2004). The aim of using various solvents is to study the selectivity and capacity of solvents to extract barley phenolic compounds (Bonoli et al 2004, Zhao et al 2006, Sun et al 2005, Liu et al 2007). Barley contains complex phenolic compounds which have activity and mechanisms that are greatly influenced by the condition of the test system. Therefore the main objectives of this study were to selectively extract, identify and quantify the major phenolic compounds from nine diverse barley genotypes employed as food, feed and malt and to determine the antioxidant capacity of the isolated extracts so obtained.

## **2.3 *Materials and Methods***

### **2.3.1 *Sample Description of the Barley Varieties***

Nine barley genotypes namely, Peru 3, Peru 16, Peru 35, EX116, EX127, EX83, CI1248, CI2230 and Hokuto Hadaka were studied. The samples are of diverse geographical origin, age

and characteristics (row type, absence or presence of hulls) and therefore representing a diverse germplasm. The 3 lines, Peru 3, Peru 16 and Peru 35 are food barleys from Peru that originated from an international centre for barley germplasm development (CYMMIT) in Mexico. The Japanese variety, Hokuto Hadaka, is widely used and has been employed in the production of miso paste. CI1248 and CI2230 are landraces collected from Israel in 1972 and Nepal in 1969 respectively. EX127 is a cross between a Brandon six-row feed line and the 19th century barley variety Faust, from Germany. Faust is a 'hooded lemma' variety that was used for grazing. EX83 is a cross between a Brandon malting line and Montcalm, a Canadian variety since 1920, grown mainly in Eastern Canada. EX116 is cross between a Moroccan landrace and the Brandon cultivar Bedford and the latter is an old feed variety.

These samples were grown in 2007 at the Agriculture and Agri-Food Canada's Brandon Research Centre located within the Barley Breeding and Genetics program, Brandon, Manitoba. All the genotypes are 6-rowed except for CI2230 which is 2-rowed. These diverse barleys were further classified according to their color based on visual appearance. The yellowed grained varieties included EX83, Ex116 and Peru 3 while the black grained varieties included Peru 35, Peru 16 and CI2230. Hokuto Hadaka, Ex127 and CI1248 showed a characteristic purple colour. Four varieties namely, Peru 3, Peru 16, Ex83 and EX116 were hulled while the rest were hull-less. Hulls were removed from husked samples prior to grain milling. Thereafter all genotypes of whole grain barley samples were ground using a Udy cyclone mill with 0.5mm sieve and stored at -20°C until further analysis. A description of the samples is shown in **Table 1**.

### **2.3.2 Chemicals**

2,2-Diphenyl-1-picrylhydrazyl (DPPH<sup>•</sup>), 2,2-azobis(2-methylpropionamide) dihydrochloride (AAPH<sup>•</sup>), monobasic potassium phosphate (KH<sub>2</sub>PO<sub>4</sub>) dibasic potassium phosphate(K<sub>2</sub>HPO<sub>4</sub>), Folin-Ciocalteu reagent, and the 4 phenolic acid standards were purchased from Sigma- Aldrich (St Louis, MO). Phenolic acid standards used for HPLC analysis included ferulic, caffeic, *p*-coumaric and sinapic. Trolox and fluorescein were purchased from Fisher Acros Organics (Fair Lawn, New Jersey) for the use with the oxygen radical absorbance capacity (ORAC). The solvents acetone, ethyl acetate, methanol, acetonitrile, acetic acid, hydrochloric acid were HPLC grade and obtained from Fisher Scientific, Fair Lawn, New Jersey. (+)-Catechin standard was purchased from Sigma-Aldrich, St Louis, MO. The sodium hydroxide and sodium carbonate was purchased from Fisher (Fair Lawn, New Jersey).

### **2.3.3 Color Measurements**

A Hunterlab spectrophotometer CM-3500d colorimeter (Minolta Co., Ltd., Osaka, Japan) with SpectraMagic version 3.6 software was used to measure the color of whole grain barley genotypes and flours. The color was expressed using the *L*, *a*, and *b* color space coordinates, where *L* represents lightness, *+a\** redness, *-a\** greenness, *+b\** yellowness, and *-b\** blueness.

### **2.3.4 Extractions of Proanthocyanidins**

Proanthocyanidin extracts were prepared according to a method by Naczki et al, (2004) as modified by Hosseinian et al, (2007). Briefly, ground barley (2.5 g) was mixed with 50 mL of acetone, water and acetic acid (70:29:1, v/v) and sonicated for 30 mins (Branson-3200R-2 sonicator, Shelton, CT). Subsequently the reaction mixture was centrifuged for 20mins at 5000 rpm (Sorvall RC5C, Sorvall instruments, DuPont, Wilmington, DE). The supernatant was

retained and used as crude extract for antioxidant assays (ORAC, DPPH, and total phenolic content (TPC)), total flavanoid content using the vanillin assay and HPLC analysis. Extractions were done in triplicates.

### **2.3.5 Extraction of Anthocyanins**

Anthocyanin extracts were prepared and purified according to Naczki et al 2004 as modified by Hosseinian et al (2008) and Bellido & Beta (2009) with some further modification. Ground barley (2.5 g) was suspended in 25 ml of acidified methanol (1N HCL, 85:15, v/v) and its pH adjusted to 1.0 using 1N HCl. The suspension was shaken for 2.5 h at room temperature (25°C) using a rotary shaker (Fermentation Design Inc, Allentown, PA) at 250 rpm and its pH corrected to 1, when necessary, 15 mins after shaking had begun. The suspension was sonicated in a Bransonic B-3200R-2 sonicator (Branson, Shelton, CT) for 30 min at room temperature and centrifuged at 10000 rpm for 30 min at 15°C using a SLA-3000 centrifuge fitted with a GS-3 rotor (Sorvall Instruments, ON, Canada). The supernatant was retained and kept at -20°C for analysis of antioxidant activity (ORAC, DPPH, and total phenolic content (TPC)) and determination of total anthocyanin content (TAC). Extractions were done in duplicates.

### **2.3.6 Extraction of Phenolic Acids**

The hydrolysis method of Krygier et al, (1982) as modified by Li et al, (2005) was further modified for this extraction. Ground barley (2 g) was hydrolyzed using 60 mL of 4M sodium hydroxide. The flask was filled with nitrogen and sealed. The mixture was stirred for 4 hrs with a magnetic stirrer plate at room temperature. Subsequently the pH of the hydrolyzed sample was adjusted to 1.5-2.5 using 6M hydrochloric acid solution and the centrifuged at 13000rpm for 40

mins (Sorvall RC5C, Sorvall Instruments, DuPont, Wilmington, DE). The supernatant was extracted four times with 70 mL of ethyl acetate. The clear extract was dried with 2 g of sodium sulphate and subsequently evaporated to dryness using a rotary evaporator set at 35°C. The residue was re-dissolved in 4 mL of 50% methanol and filtered through a 0.45 µm nylon filter. The extract was stored at -20°C and used for antioxidant assays (ORAC, DPPH, and total phenolic content (TPC)) and HPLC analysis.

### **2.3.7 Antioxidant Assays**

#### **2.3.7.1 Total Phenolic Content (TPC)**

The total phenolic content of barley extracts was determined according to a laboratory procedure using the Folin-Ciocalteu method (Singleton and Rossi 1965). Briefly, a barley extract (200µL) was added to 1.5 mL of 10 fold diluted and freshly diluted Folin-Ciocalteu reagent freshly prepared and equilibrated for 5 minutes after vortexing. Subsequently, 1.5 mL of sodium carbonate (60 g/L) was then added to neutralise the mixture which was then vortexed. After 90 mins of reaction at ambient temperature, absorbance was measured at 725 nm against reagent blank and used to calculate the total phenolic content using ferulic acid as a standard. Concentrations ranging from 100-1000 µM of ferulic acid were used for quantification. Analyses were conducted in triplicates. The results were expressed as ferulic acid equivalents on a dry matter basis.

#### **2.3.7.2 DPPH**

The DPPH method of Brand- William et al (1995) as modified by Li et al (2005) was further modified for this assay. Briefly, a 60µM DPPH radical solution was freshly prepared from a

stock solution of 300  $\mu\text{M}$ . Barley extracts (100  $\mu\text{L}$ ) were reacted with 3.9 mL of the DPPH radical solution for 60mins. The absorbance at 515nm was measured against a blank of pure methanol at  $t = 0, 5, 10, 20, 30, 40, 50$  and 60 mins and used to estimate the level of free radical scavenging ability. Concentrations ranging from 100 to 1000  $\mu\text{M}$  were used for the trolox standard curve. Antioxidant activity was calculated as a percentage DPPH radical scavenging activity expressed using Trolox equivalents (TE) per gram of dry matter (DM) using the following equation;

$$\% \text{ DPPH scavenging activity} = \left(1 - \frac{A_{\text{sample},t}}{A_{\text{control},t}}\right) \times 100$$

where  $A_{\text{sample},t}$  is the absorbance of the sample at time = 30 mins and  $A_{\text{control},t}$  is the absorbance of the control at time = 0 mins.

### **2.3.7.3 ORAC**

The determination of oxygen radical absorbance capacity (ORAC<sub>FL</sub>), first developed by Cao et al 1993 was conducted according to Huang et al 2002 and Li et al 2007. An FLx800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT) was used with fluorescence filters for an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm. The plate reader was controlled by KC4 3.0 software (version 29). Dilution of barley extract, catechin control, and Trolox standard was done manually. The quantity of 300  $\mu\text{L}$  each of buffer solution (blank) and diluted sample solution, catechin control, and Trolox standard was transferred to a 96-well flat bottom polystyrene microplate (Corning Incorporated, Corning, NY, USA) by hand according to their allocated wells. Trolox standards of concentrations 12.5, 25, 50

and 100  $\mu\text{M}$  were used. A full automation of plate-to-plate liquid transfer was programmed by using a Precision 2000 automated microplate pipetting system (Bio-Tek Instruments, Inc., Winooski, VT). Specifically, 120  $\mu\text{L}$  of fluorescence working solution was transferred from the reagent holder to each well of a second 96-well microplate. Then 20  $\mu\text{L}$  each of buffer solution (blank), Trolox standard, diluted samples, and 20 $\mu\text{M}$  catechin control from designated wells of the first 96-well microplate were transferred to designated wells of the second 96-well microplate. The latter was quickly covered with an adhesive sealing film, shaken for 3 min at 37  $^{\circ}\text{C}$  in the microplate reader, and incubated in the preheated (37 $^{\circ}\text{C}$ ) microplate reader for a total period of 15 min. The second 96-well microplate was transferred back to its original station in the Precision 2000 automated microplate pipetting system, followed by automatically transferring 60  $\mu\text{L}$  of AAPH solution from the reagent holder to designated wells. Thus, the total volume for each well was 200  $\mu\text{L}$ . The second 96-well microplate was quickly covered again with an adhesive sealing film and immediately transferred to the microplate reader, and the fluorescence was measured every minute for 50 min at 37  $^{\circ}\text{C}$ . Peroxyl radical was generated by AAPH during measurement, and fluorescein was used as the substrate. All reaction mixtures were prepared in the measured plate in quadruplates, and at least two independent assays were performed for each sample.

Data was processed according to Cao et al 1993 and Huang et al 2002. The final ORAC values were calculated by using a linear regression equation between the Trolox concentration and the net area under the fluorescence decay curve. Area under curve (AUC) was calculated as follows:

$$\text{Net AUC} = \text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}$$

The area under the fluorescence decay curve was calculated as follows;

$$\text{AUC} = 0.5 + f_1/f_0 + f_i/f_0 + \dots + f_{49}/f_0 + 0.5(f_{50}/f_0)$$

where  $f_0$  = initial fluorescence reading at 0 mins and  $f_i$  = fluorescence reading at time  $i$  min.

The net AUC was obtained by subtracting the AUC of the blank from that of the sample as shown above. The final results (ORAC value) were calculated and expressed using Trolox equivalents per 100 grams dry weight basis.

### **2.3.8 Determination of Proanthocyanidin Content**

The proanthocyanidin content was determined according to Price et al (1978) as modified by Sun et al (1998) with further modifications. Proanthocyanidin extracts (1 mL) were reacted with 5ml of vanillin reagent (50:50 mixture with 1% vanillin in acetic acid/ 8% HCl in acetic acid) for 20 mins at room temperature and the absorbance read at 510 nm. Catechin was used as the standard with concentration ranging from 100 to 700  $\mu\text{g/ml}$  and the results were expressed as  $\mu\text{g}$  catechin equivalents (CE)/g of barley on dry weight basis after calculation using the following equation obtained from the standard curve;  $y = 0.0013x + 0.0244$ , where  $y$  is absorbance at 510 nm and  $x$  is concentration of catechin standard.

### **2.3.9 Determination of Total Anthocyanin Content**

The determination of the total anthocyanin content (TAC) was done using the spectrophotometric method reported by Abdel-Aal et al, (1999) as modified by Siebenhandl et al, (2007). Briefly, ground barley (1 g) was mixed with 10 mL acidified methanol (methanol and 1N

HCl, 85:15, v/v). The solution was mixed and adjusted to pH 1 using 1N HCl and shaken for 2.5 hours at room temperature. Subsequently the reaction mixture was centrifuged for 20 mins at 13000 rpm at 15°C and the supernatant was retained for the spectrophotometric assay. The absorbance of the resulting extract was measured at 535 nm against a reagent blank. TAC was expressed as microgram cyanidin-3-glucose per gram of whole barley (dry weight basis) and calculated using the equation shown below;

$$C = (A/\epsilon) \times (\text{vol}/1000) \times 449 \times MW \times (1/\text{Sample weight}) \times 10^6$$

where C is the concentration of the total anthocyanin ( $\mu\text{g}/\text{g}$ ), A is the absorbance reading,  $\epsilon$  is molar absorptivity (cyanidin-3-glucoside = 25 965), vol is total volume of anthocyanin extract and MW is the molecular weight of cyanidin-3-glucoside = 449.

### ***2.3.10 HPLC-ESI-MS Analysis of Proanthocyanidins and Phenolic Acids***

A 2696 HPLC system (Waters Corp., Milford, MA) equipped with a model 996 photodiode array detector (PDA) (Waters) and model 717 plus autosampler (Waters) coupled with a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micro Mass Waters, Milford, MA) was employed for HPLC-MS analysis with negative electrospray ionization. A 150 mm  $\times$  4.6 mm, 5  $\mu\text{m}$  RP 18 analytical column was used for separation (Gemini, Phenomenex, Torrance, CA). During the LC/MS analysis, 10  $\mu\text{l}$  of sample was loaded and injected by an autosampler, and eluted through the column with gradient mobile phase, then introduced to the Q-TOF. For the analysis of proanthocyanidins (PA), the mobile phase A was made up of water containing 0.5% acetic acid and mobile phase B was made up of acetonitrile containing 0.5% acetic acid. The 75 min-linear gradient for PA quantification was programmed as follows: 0-10 min, 5-10% B; 10-

30 min, 10-15% B; 30-45 min, 15-20% B; 45-60 min, 20-60% B; 60-65 min, 60% B; 65-70 min, 60-5% B; 70-75 min 5% B. On the other hand, mobile phase A for phenolic acids consisted of water containing 0.1% acetic acid and mobile phase B consisted of methanol containing 0.1% acetic acid. The 75 min-linear gradient for phenolic acids quantification was programmed as follows: 0-7 min, 15-20% B; 7-8 min, 20-15% B; 8-21 min, 15-24% B; 21-34 min, 24-13% B; 36-37 min, 13-20% B; 37-46 min, 20-42% B; 46-63 min 42-100% B; 63-75 mins, 100-15%B. Flow rates were set at 0.5 ml/min. The full mass spectra were recorded in negative mode by using the capillary voltage of 1.2 kV and cone voltage of 40 V. The flow rate of desolvation gas (N<sub>2</sub>) and cone gas were 900 L/h and 50 L/h, respectively. The desolvation temperature and the source temperature were set at 350°C and 150°C, respectively. The MS/MS spectra were acquired by using collision energy of 30 V. The absorbance at 280 nm and 310 nm was used for quantification of the PA and phenolic acids respectively. Vanillic acid was quantified at 291nm as it showed maximum absorption at that wavelength. Catechin was the external standard for quantification of flavan-3-ols while ferulic, sinapic, caffeic, vanillic and p-coumaric acid were used as external standards for quantification of phenolic acids. Ferulic acid dehydrodimers were quantified according to a method by Waldron et al (1996) using the following response factors (RFs) against trans cinammic acid at 280nm: RF 0.14 for 8-O-4' DFA and RF 0.12 for 8-5' DFA benzofuran form.

### ***2.3.11 Statistical Analysis***

Results based on triplicate analyses of barley genotypes were reported as mean  $\pm$  standard deviation. The data from each assay was analyzed separately by one way analysis of variance (ANOVA) using the general linear model (GLM) of SAS software package (release 9.1) (SAS

Institute, Cary, NC). Significant differences were detected at  $p < 0.05$  and determined by comparing the difference between means using the least square difference (LSD). Correlation analyses were performed with the PROC CORR procedure of the SAS software package using the Pearson correlation test.

## **2.4 Results and Discussion**

### **2.4.1 Hunterlab Spectrophotometer Colour Measurements**

Colour and appearance of food barleys have implications on the end quality and perception of whole grain food products. Based on visual appearance, these food barleys were classified into three categories: black grained, yellowed grained and purple grained. The Hunterlab spectrophotometric assay further confirmed the colors that were observed. CI-1248, Hokuto Hadaka and EX127 were of mixed appearance and were classified as purple. The light yellow grain (or normal barley) group comprised of EX-83, EX116 and PERU 3. Peruvian genotypes (Peru 35 and Peru 16) and CI2230 were black in color. This black pigmentation may be attributed to the presence of melanin-like pigmentation that may overlap purple or blue color as a result of the anthocyanins (Siebenhandl et al 2007).

The L, a\*, b\* values show degree of lightness, redness and yellowness respectively. Results corresponding to L, a\* and b\* values for the nine barley varieties (grains and flours) are shown in **Table 2.1**. The L value which measures the degree of lightness was lowest for the black barley grains (L=29.20, CI2230), highest for yellow grains (L=58.92, EX116) and intermediate for the purple grains (L=45.23, EX127). The a\* values (redness) were highest in the grains and flours of the purple barley group (CI-1248, Hokuto Hadaka and EX127). A similar

observation relating to the degree of redness and grain color was reported by Kim et al 2007 in a total of 127 barley lines they analyzed and Bellido & Beta 2009 who used 3 genotypes (Peru 35, CI1248 and EX 83) included in this study. The L and b\* values for the grains and flours respectively had a high positive correlation ( $r=0.869$ ,  $p=0.02$ ) and ( $r=0.54$ ,  $p=0.13$ ). Kim et al 2007 reported that barley can be classified as black, blue, and purple on the basis of lemma, pericarp, and aleurone color.

**Table 2.1: Sample Description and Hunter color L, a\* and b\* Values for Nine Barley Grains Ground into Flour**

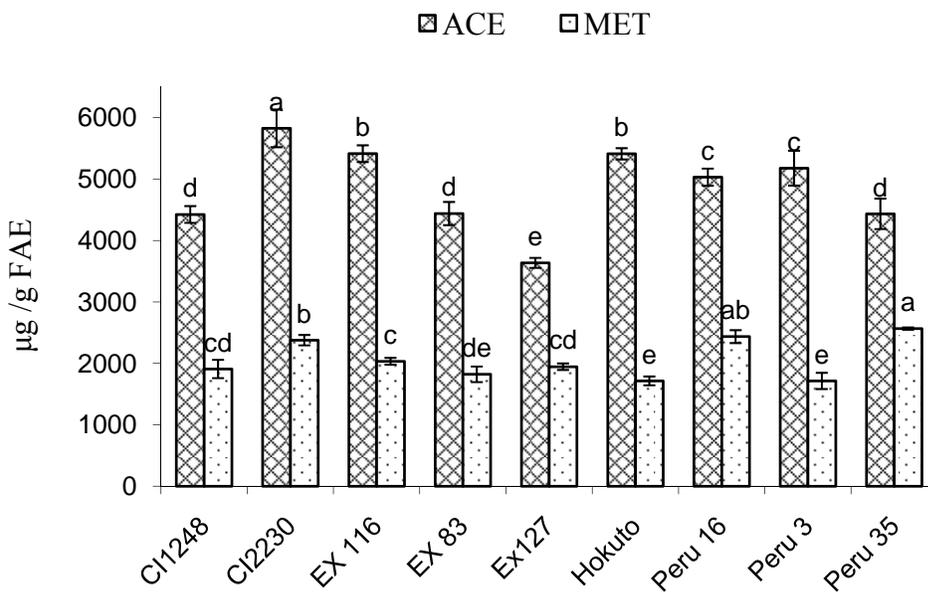
<b>Barley Genotype</b>	<b>L*</b>	<b>a*</b>	<b>b*</b>	<b>L*</b>	<b>a*</b>	<b>b*</b>	<b>Colour</b>	<b>Type of Caryopsis</b>	<b>Row Type</b>	<b>Origin</b>
	<b>Grain</b>			<b>Flours</b>						
CI1248	43.56	7.19	20.01	83.45	1.81	8.98	Purple	Hull-less	6	Israel
CI2230	29.90	2.17	7.34	77.22	0.72	6.02	Black	Hull-less	2	Nepal
EX 116	58.92	4.94	34.08	90.84	0.61	8.44	Yellow	Hulled	6	Mix( Moroccan & Canadian)
EX 83	64.07	4.45	23.62	89.93	0.79	9.05	Yellow	Hulled	6	Mix (Canadian)
Ex127	45.23	7.02	22.36	83.21	2.02	11.08	Purple	Hull-less	6	Mix (Canadian & Germany)
Hokuto Hadaka	52.94	7.30	25.06	85.43	1.86	11.37	Purple	Hull-less	6	Japan
Peru 16	39.82	2.03	8.58	78.54	0.87	5.44	Black	Hulled	6	Peru
Peru 3	55.76	4.63	28.63	89.48	0.64	8.26	Yellow	Hulled	6	Peru
Peru 35	32.78	3.54	10.71	76.84	1.15	6.81	Black	Hull-less	6	Peru

## ***2.4.2 Antioxidant Capacities of the Barley Genotypes***

### ***2.4.2.1 Total Phenolic Content of the Barley Genotypes***

The total phenolic content can be used as a measure of reducing capacity of an extract and thereby correlated to antioxidant capacity. Using the Folin- Ciocalteau method, the total phenolic content was expressed as ferulic acid equivalents (FAE). The total phenolic content (TPC) for the methanolic extracts ranged from 1714 (Peru 3) – 2566 (Peru 35)  $\mu\text{g/g}$  (**Table 2.2**) while the acetone extracts ranged from 3636 (EX127) – 5826 (CI2230)  $\mu\text{g/g}$  (**Table 2.3**). The TPC for the alkali hydrolyzed extracts ranged from 785 (EX116) – 1403 (EX127)  $\mu\text{g/g}$  (**Table 2.4**). Acetone extracts (70% acetone) exhibited the highest total phenolic content showing the selectivity and capacity of solvent to extract the major phenolic compounds of barley (

**Figure 2.1).**



**Figure 2.1: Total phenolic content (TPC) for the aqueous methanolic and acetone extracts of different barley varieties ( $\mu\text{g/g}$  FAE). MET; Methanolic extract, ACE; Acetone extract.**

*Methanolic Extract* Acidified methanol extracts showed the ability of phenolic compounds to reduce the Folin reagent as shown in **Table 2.2**. The barleys were categorised as follows: genotypes (CI2230, Ex116, Peru 16 and Peru 35) with TPC greater than  $2000 \mu\text{g/g}$  were regarded as high TPC barleys, those with TPC between  $1900 \mu\text{g/g}$  and  $2000 \mu\text{g/g}$  were considered medium TPC barleys and genotypes with less than  $1900 \mu\text{g/g}$  were classified as low

TPC barleys. Peru 35 (2566  $\mu\text{g/g}$ ) had the highest TPC in its methanolic extract; however there was no significant difference between Peru 35 and Peru 16 (2436  $\mu\text{g/g}$ ). The Peruvian barleys (Peru 35 and Peru 16) showed significantly higher TPC ( $p < 0.05$ ) than the other genotypes. There was no significant difference between Peru 16 and CI2230. Peru 35, Peru 16 and CI2230 were classified as black grains. The pigmented genotypes are likely to contain high contents of polyphenolic compounds. Choi et al (2007) also reported high percentage scavenging activity of methanolic extracts from pigmented grains (black rice, red sorghum, brown rice and barley) on DPPH radical. Peru 3, EX83 and Hokuto Hadaka had no significant differences in the TPC and were classified as low TPC barleys; however, Peru 3 had the least TPC among the 9 barleys. CI1248 and EX 127 were classified as medium TPC barleys accordingly. Previous studies by Quinde-Axtell et al (2006) found that the amount of phenolic compounds varied according to presence or lack of hulls. Among barley genotypes, hull-less CI2230, CI1248 and Peru 35 had significantly high TPC. However, the high TPC present in the hull-less genotypes was also found associated with the hulled genotypes, Peru 16 and EX116.

*Acetone Extract* The genotypes were categorized according to levels of TPC as follows: genotypes with TPC greater than 5000  $\mu\text{g/g}$  were regarded as high TPC barleys, those with TPC between 4000  $\mu\text{g/g}$  and 5000  $\mu\text{g/g}$  were considered medium and genotypes with less than 4000 were classified as low TPC barleys. In the acetone extract CI2230, originating from Nepal had the highest reducing capacity followed by EX116 and Hokuto Hadaka. EX127 (3636  $\mu\text{g/g}$ ), a cross between Brandon six-row feed line and the 19th century barley variety Faust, from Germany displayed the lowest TPC as contents were below 4000  $\mu\text{g/g}$ . Aqueous acetone was employed to target the extraction of flavan-3-ols therefore the total phenolic content of the

acetone extracts are likely mainly due to the PAs as these are the main compounds present in the crude extract (**Table 2.3**). CI1248 (4424  $\mu\text{g/g}$ ), Peru 35 (4435  $\mu\text{g/g}$ ) and EX83 (4439  $\mu\text{g/g}$ ) exhibited medium TPC and did not differ significantly. There were no significant differences between Peru 3 (5178  $\mu\text{g/g}$ ) and Peru 16 (5031  $\mu\text{g/g}$ ) which were categorized as high TPC barleys.

The variation in the reducing capacity of the barley genotypes can be explained by the variation in the contents of phenolic compounds such as flavonoids with different structures that are present in the extracts. Depending on their respective structures, some phenolic compounds can be weak or strong electron donors and some develop synergistic and antagonistic effects with themselves and other compounds existing in the extract (Rice-Evans et al 1996). Researchers have demonstrated that aqueous acetone is more selective and efficient in extracting flavanols (Bonoli et al 2004, Zhao et al 2008, Quinde- Axtell et al 2006, Liu et al 2007).

*Alkaline Hydrolyzed Extract* The total phenolic content assay was also conducted for the alkali hydrolyzed extracts and contents ranging from 785 (EX116) to 1403 (EX127)  $\mu\text{g/g}$  were found. The barleys were classified according to their respective TPC as follows: genotypes with TPC greater than 1000  $\mu\text{g/g}$  were regarded as high, those with TPC between 900  $\mu\text{g/g}$  and 1000  $\mu\text{g/g}$  were considered medium and genotypes with less than 900  $\mu\text{g/g}$  were classified as low TPC barleys. EX127 (1403  $\mu\text{g/g}$ ), a cross between a Canadian and German barley genotypes had the highest phenolic content and also exhibited the highest antioxidant properties as well (**Table 2.4**). The high TPC and phenolic acids associated with the genotype EX127 prompts the likelihood that the reducing capacity of the extract is attributed to the phenolic acids present in

the alkali hydrolyzed extract. Peru 16 (1269  $\mu\text{g/g}$ ) and CI1248 (1178  $\mu\text{g/g}$ ) had no significant differences in their TPC. Peru 35 (1084  $\mu\text{g/g}$ ) and CI2230 (1025  $\mu\text{g/g}$ ) also showed no significant differences in their TPC. Peru 16, CI1248, Peru 35 and CI2230, all dark colored grains, were classified as high TPC barleys using their alkaline hydrolyzed extracts. The yellow grained, Peru 3 (828  $\mu\text{g/g}$ ) and EX116 (785  $\mu\text{g/g}$ ), had no significant differences being categorized as low TPC barleys.

Zhao et al (2008) have reported total phenolic contents ranging from 2170 to 2560  $\mu\text{g}$  GAE /g dry basis in a study conducted with 14 barley varieties, while Dvorakova et al (2008) found ranges between 211 to 606 mg GAE /kg dry weight basis in a study with 10 barley varieties and 1030 (water extract) to 1870 (80% acetone extract) mg GAE /g (Zhao et al 2006) in a study with 3 barley varieties. The variation in the literature results is likely due to difference in genotypes, analytical methods and the use of different standards. Ranges between 1709 and 1990  $\mu\text{g}$  FAE/g have also been reported for 6 wheat genotypes (Mpofu et al 2006). The original TPC assay by Singleton et al (1965) used gallic acid as a standard, however, other standards such as catechin, tannic acid, chlorogenic acid and caffeic acid have also been used (Karadag et al 2009). Ferulic acid, the major monomeric phenolic acid in barley was used as a standard for the TPC assay (Hernaz et al 2001; Holtekjolen et al 2006; Nordkvist 1984). Gallic acid in barley has been reported in low contents in water extracts (Zhao et al 2006). The use of gallic acid as standard for TPC in barley is likely to result in low estimation. The absorbance values obtained for the TPC assay depend on number of hydroxyl groups and molecular structure of phenolic compound (Stratil et al 2006). In a study by Stratil et al (2006), the absorbance values for caffeic acid (two reacting OH) is approximately twice and for catechin (three reacting OH) three times

higher than that for phenol (one reacting OH). There is a high likelihood that a highly reactive standard will give high absorbance and this will result in low values being measured in the samples. In comparison with other barley grains in literature, the nine genotypes used in this study generally had high total phenolic content. Differences in TPC between the different extracts can be attributed to the selectivity and extraction capacity of solvents for specific barley phenolic compounds. Aqueous acetone is aimed at extracting flavanols, acidified methanol targets anthocyanins and the alkali hydrolysis releases the phenolic acids. Furthermore, this assay accounts for the transfer of electrons from phenolic compounds and other reducing species to molybdenum under basic condition (Singleton et al 1965).

**Table 2.2: Antioxidant Activity and Total Anthocyanin Content of the Methanolic Extracts of the Nine Barley Genotypes.**

Barley Genotype	Colour of Extract	TAC (mg/kg)	TPC (FAE µg/g)	ORAC (TE µmol/100g)	DPPH (TE µmol/100g)	% Radical Scavenging Activity
CI1248	Light red	37.8±0.42a	1909±149cd	4260±302d	1112±11.02ab	76.18
CI2230	Orange-Brown	27.4±0.64c	2378±85b	5278±213b	1074±12.05cd	73.82
EX 116	Rose pink	11.9±0.34f	2034±56c	4691±333c	910±10.88f	62.51
EX 83	Rose pink	14.1±0.38e	1822±127de	5246±148b	1057±10.80d	71.66
Ex127	Pink	19.0±0.05d	1946±53cd	4870±187c	1106±9.53ab	75.82
Hokuto	Golden yellow	8.3±0.14g	1715±72e	4092±146d	1115±15.46ab	75.76
Peru 16	Orange-Brown	30.6±1.94b	2436±105ab	5882±623a	1094±27.35bc	75.00a
Peru 3	Rose pink	14.7±0.20e	1714±132e	3492±353e	1122±7.75a	76.46
Peru 35	Dark yellow	13.1±0.23ef	2566±18a	4718±155c	945±11.81e	64.08
LSD	-	1.63	167.16	360.4	24.12	-

Mean values of samples having similar letters are not significantly different (P<0.05);

TE, Trolox Equivalent

TAC, Total Anthocyanin content

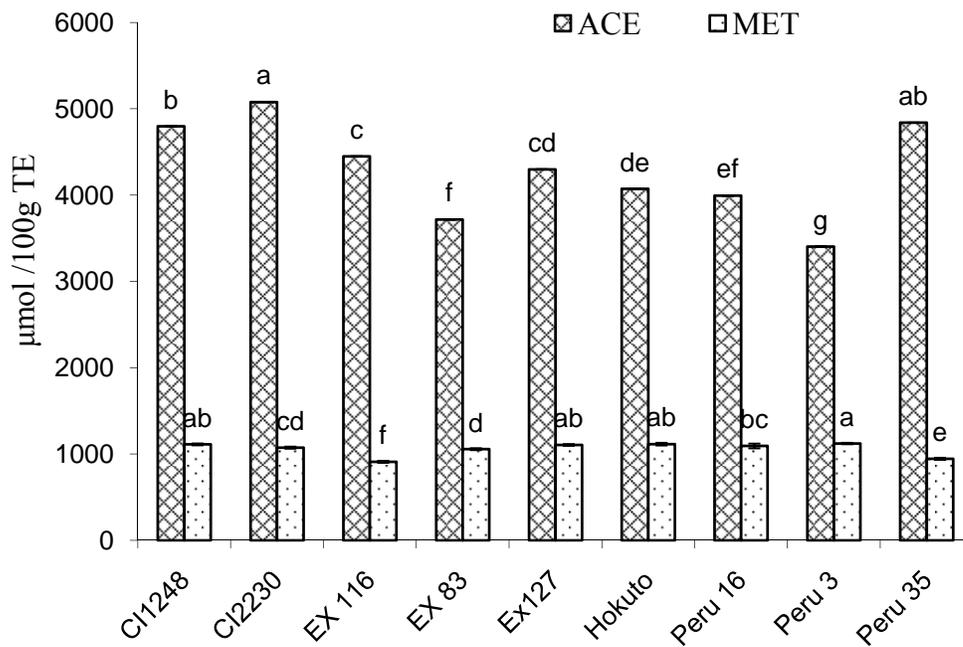
TPC, Total Phenolic Content

FAE, Ferulic Acid Equivalent

LSD denotes fisher's least significant difference.

#### **2.4.2.2 DPPH Radical Scavenging Activities of the Barley Genotypes**

DPPH assay measures the reducing ability of antioxidants towards DPPH radical. The radical scavenging activity was measured as percentage discoloration and also expressed as trolox equivalents per 100 g dry weight basis. In the methanolic, acetone and alkali hydrolyzed extracts, the radical scavenging activity ranged from 910 (Ex116) to 1122 (Peru 3)  $\mu\text{mol TE}/100\text{g}$  (**Table 2.2**), 3405 (Peru 3) to 5078 (CI2230)  $\mu\text{mol TE}/100\text{g}$  (**Table 2.3**) and 360 (EX116) to 538 (EX127)  $\mu\text{mol TE}/100\text{g}$  (**Table 2.4**) respectively. The DPPH radical scavenging capacity of the alkali hydrolyzed extract is mainly attributed to the presence of phenolic acids. Compared to the methanolic and acetone extracts, the alkali hydrolyzed extracts showed lower radical scavenging activity and this can be attributed to the presence of stronger antioxidants such as flavonoids in the methanol and acetone extracts. The values obtained using the DPPH method for the nine barley genotypes in acidified methanol and aqueous acetone are displayed in **Figure 2.2**. The methanolic extract showed about one-fifth radical scavenging activity of the acetone extract. The acetone extract exhibited the highest DPPH scavenging activity and TPC.



**Figure 2.2: 2, 2-Diphenyl-picrylhydrazyl radical scavenging activity (DPPH) for the aqueous methanolic and acetone extracts of different barley varieties ( $\mu\text{mol}/100\text{g TE}$ ). MET; Methanolic extract, ACE; Acetone extract.**

*Methanolic Extract* In the methanolic extract the DPPH radical scavenging activity ranged from 62 to 75 %. Peru 3, a yellow-hulled genotype had the highest radical scavenging activity among the methanolic extracts. No significant differences were found among the purple coloured genotypes, CI1248 (1112  $\mu\text{mol}$ ), EX127 (1106  $\mu\text{mol}$ ) and Hokuto Hadaka (1115  $\mu\text{mol}$ ). Hokuto Hadaka also had relatively high scavenging activity. EX116 (910  $\mu\text{mol}$ ), a yellow grained variety had the least DPPH scavenging activity. Peru 35 and EX116 had relatively low radical scavenging activity while EX83 and CI2230 had medium radical scavenging activity.

Among the Peruvian genotypes, Peru 3 and Peru 16 were significantly high in their ability to scavenge the DPPH free radical. The black grains, CI2230 and Peru 16 had no significant differences in their radical scavenging capacity in the methanolic extract. Kim et al (2007) used 127 colored barley samples to determine the radical scavenging activity and the values had a wider range from 46.6 to 86.3 % for methanolic extracts.

*Acetone Extract* The nine barley genotypes displayed a radical scavenging activity varying from 3405 to 5078  $\mu\text{mol TE}/100\text{ g dry basis}$ , about 3.5 to 4 times more than reported by Zhao et al (2008). Zhao et al (2008) studied the radical scavenging capacity of 14 barley samples using the DPPH assay and found that the samples ranged from 933 to 1178  $\mu\text{mol TE}/100\text{ g dry basis}$ . A different ranking from the methanolic extracts was observed for the acetone extract. CI2230 (5078  $\mu\text{mol}$ ), a Nepalese black grain, exhibited the highest radical scavenging property followed by Peru 35 (4840  $\mu\text{mol}$ ), CI1248 (4798  $\mu\text{mol}$ ), EX116 (4449  $\mu\text{mol}$ ) and EX127 (4299  $\mu\text{mol}$ ). No significant differences were observed between Peru 35 and CI2230 and between Peru 35 and CI1248. The hull-less barley genotypes (CI2230, CI1248, EX127) showed relatively high DPPH radical scavenging activity. Other researchers have reported high contents of flavanols in hull-less barley varieties (Dvorakova et al 2008; Holtekjolen et al 2006; Quindell-Axtell et al 2006). The barley genotype with the least DPPH radical scavenging activity was Peru 3 (3405  $\mu\text{mol}$ ). A low DPPH radical scavenging capacity gives an indication that the DPPH radical will only react with the more reactive phenolic compounds in the extracts since the DPPH radical is very stable (Stratil et al 2007). Peru 3 had the lowest DPPH radical scavenging activity for the acetone extracts but had the highest in the methanolic extract. Different phenolic

compounds have structural variation and hence they show varied reactivity with free radicals (Holtekjolen et al 2006; Rice-Evans et al 1997).

*Alkaline Hydrolyzed Extracts* Among the hydrolyzed extracts, purple-grained EX127 (538  $\mu\text{mol}$ ) exhibited the highest radical scavenging activity while yellow- grained EX116 (360  $\mu\text{mol}$ ), had the lowest. CI1248 (481  $\mu\text{mol}$ ), a purple grained genotype from Israel had the second highest radical scavenging activity. No significant difference was observed between Hokuto Hadaka and CI1248. Interestingly, Peru 16 (404  $\mu\text{mol}$ ), Peru 3 (399  $\mu\text{mol}$ ) and Peru 35 (421  $\mu\text{mol}$ ) had no significant differences in their DPPH radical scavenging capacity for the alkali hydrolyzed extracts and were classified as having medium scavenging activity. Significantly low radical scavenging activity was exhibited by EX116, EX83 and CI2230. The hydrolyzed extracts had lower antioxidant capacity compared to the acetone and acidified methanolic extracts. This can be explained by the composition of phenolic compounds in the respective extracts. The acetone extracts comprises of flavanols while the extraction with acidified methanol mainly targets anthocyanins; however in both methanol and acetone extractions, other free phenolics may be liberated (Zhao et al 2006). Alkaline hydrolysis was aimed at releasing the bound phenolic acids. Generally, polyphenols are stronger antioxidants than monophenols in lipid systems (Rice- Evans et al 1996) and using chemical assays such as DPPH radical scavenging activity (Brand-Williams et al 1995). Holtekjolen et al 2006 and Brand-Williams et al 1997 reported that the methoxy substitution can increase the antioxidant capacity of monophenols; however, for the phenolic acids this effect is not as important compared to the addition of hydroxyl groups.

Various researchers have used the DPPH assay as a tool to quantify the antioxidant capacity using different protocols thereby making the comparison of results difficult. A good DPPH assay should take into account spectrophotometric sensitivity range, sensitivity of DPPH to light, pH and solubility of the compound (Huang et al 2005). There is a variety of phenolic compounds found in barley extracts such as phenolic acids, proanthocyanidins and anthocyanins and these compounds have various DPPH scavenging activities as a function of their chemical structures (Holtekjolen et al 2006). The higher DPPH radical scavenging activity displayed in the crude acetone extracts suggests that flavanols have higher antioxidant activity in comparison to phenolic acids in the alkaline hydrolyzed extracts and anthocyanins that are likely present in the methanolic extracts.

**Table 2.3: Antioxidant Activity and Total Proanthocyanidin Content of the Acetone Extracts of the Nine Barley Genotypes.**

Barley Genotype	Proanthocyanidin Content (CE µg/g)	TPC (FAE, µg/g)	ORAC (TE, µmol/100g)	DPPH (TE, µmol/100g)	% DPPH Radical Scavenging Activity
CI1248	843±38b	4424±137d	6594±285e	4798±3.19b	53.66
CI2230	1029±51a	5826±307a	5596±492e	5078±0.90a	56.67
EX 116	843±35b	5414±136b	6488±116d	4449±1.10c	49.65
EX 83	441±3f	4439±189d	6815±271d	3718±1.39f	41.88
EX127	550±21e	3636±82e	7892±330c	4299±1.87cd	48.49
Hokuto Hadaka	709±11c	5412±92b	5782±221e	4074±1.72de	45.73
Peru 16	726±8c	5031±139c	9321±245a	3994±0.40ef	44.73
Peru 3	640±17d	5178±284bc	5912±283e	3405±1.17g	38.92
Peru 35	987±81a	4435±248d	8853±72b	4840±1.01ab	53.13
LSD	64.67	335.15	408.59	264.15	-

Values expressed as mean ± standard deviation, LSD denotes Fisher's least significant difference.

Mean values of samples having similar letters are not significantly different (P<0.05).

TE, Trolox Equivalent

TPC, Total Phenolic Content

FAE, Ferulic Acid Equivalent.

### 2.4.2.3 ORAC Values of the Barley Genotypes

ORAC, a hydrogen atom transfer mechanism-based assay, was used to measure the inhibition of peroxy radical-induced oxidations. The ORAC values are expressed as  $\mu\text{mol}$  of trolox equivalents per 100 grams on dry weight basis. ORAC values for the nine barley genotypes in acidified methanol and aqueous acetone are displayed in Error! Reference source not found.. In the methanolic, acetone and alkaline hydrolyzed extracts the ORAC values ranged from 3492 (Peru 3) to 5882 (Peru 16)  $\mu\text{mol TE}/100\text{g}$  (**Table 2.2**), 5596 (CI2230) to 9321 (Peru 16)  $\mu\text{mol TE}/100\text{g}$  (**Table 2.3**) and 3441 (CI2230) to 4612 (EX127)  $\mu\text{mol TE}/100\text{g}$  (**Table 2.4**) respectively. There were significant differences in ORAC values among the extracts from barley genotypes.

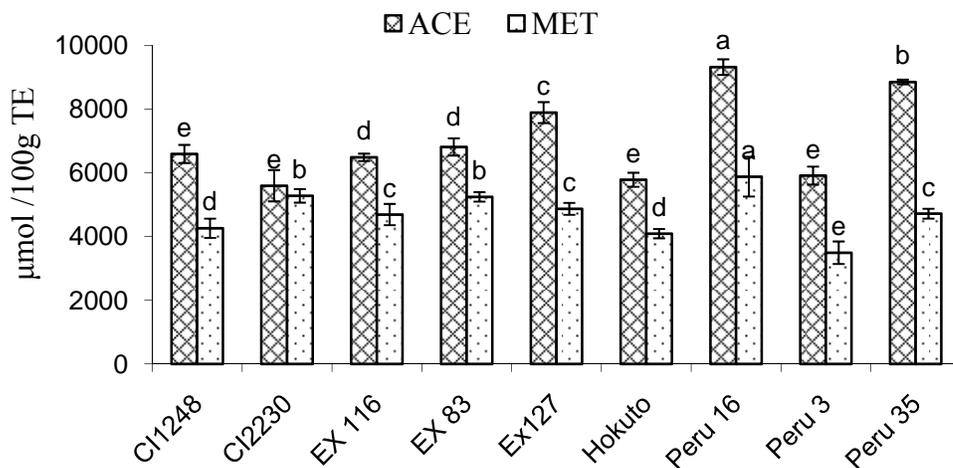


Figure 2.3: Oxygen Radical Absorbance Capacity (ORAC) values for the aqueous methanolic and

**acetone extracts of different barley varieties ( $\mu\text{mol}/100\text{g TE}$ ). MET; Methanolic extract, ACE; Acetone extract.**

*Methanolic Extract* In reference to the methanolic extract, Peru 16 had the highest ORAC value. There were no significant differences in ORAC values for CI2230 (5278  $\mu\text{mol}$ ) and EX 83 (5246  $\mu\text{mol}$ ) and between EX116 (4691  $\mu\text{mol}$ ) and EX127 (4870  $\mu\text{mol}$ ). The ORAC assay measures the ability of the phenolic compounds in the extract to scavenge for the peroxy radicals. EX116, EX127 and Peru 35 had relatively high ability to quench the peroxy radicals as exhibited by the ORAC values and there were no significant differences among their antioxidant activities. Generally, the dark colored genotypes namely, Peru 16, CI2230 and Peru 35 had relatively high antioxidant activity. Similar observations were made with the DPPH and TPC assays. Peru 35 and Peru 16 exhibited exceptional antioxidant properties as measured by DPPH, TPC and ORAC for the methanolic extracts.

*Acetone Extract* Ranking of antioxidant activity based on the acetone extract were in following decreasing order: Peru16 > Peru 35 > EX127 > EX83 > CI1248 > EX116 > Peru 3 > Hokuto Hadaka > CI2230. Peru 16 exhibited high ORAC value for acetone extract implying free phenolics liberated by these solvents reacted readily with the peroxy radicals. There were no significant differences among the genotypes, Hokuto Hadaka, Peru 3, CI1248 and CI2230. Interestingly, the Nepalese genotype, CI2230 which showed high antioxidant capacities with DPPH and Folin assays had the least ORAC value for the acetone extract. Both yellow-colored grains, EX116 (6488  $\mu\text{mol}$ ) and EX83 (6815  $\mu\text{mol}$ ) had no significant differences in the ORAC values ( $p > 0.05$ ). The high ORAC values obtained from the acetone extract can be attributed to the flavanols liberated by this solvent during extraction that have the ability to donate hydrogen

atoms (Zhao et al 2006). During the ORAC assay free peroxy radicals are constantly being generated by 2,2'-azobis (2-methyl propionamide) dichloride (AAPH). This unique method results in a complete reaction with a combination of inhibition time and level of inhibition (Tabart et al 2009).

*Alkaline Hydrolyzed Extracts* Among the hydrolyzed extracts, EX127 (4612) had the highest ORAC values. In fact this barley variety was the highest in all the antioxidant assays for the alkaline hydrolyzed extracts. The dark colored grains, Peru 35, Peru16, and Hokuto Hadaka showed no significant differences among them. The lowest ORAC value for the hydrolyzed extracts was found in CI2230 (3441  $\mu\text{mol}$ ). Ranking of antioxidant activity based on the hydrolyzed extract for the ORAC assay was as follows: EX127 > Peru 35 > Peru 16 > Hokuto Hadaka > CI1248 > Peru 3 > EX83 > EX116 > CI2230. According to the ORAC assay the Peruvian genotypes displayed relatively high ORAC values and no significant differences were shown between Peru 35(4217  $\mu\text{mol}$ ) and Peru 16 (4574  $\mu\text{mol}$ ).

**Table 2.4: Antioxidant Activity of the Alkali Hydrolyzed Extracts of the Nine Barley Genotypes**

Barley Genotype	TPC (FAE $\mu\text{g/g}$ )	ORAC (TE $\mu\text{mol}/100\text{g}$ )	DPPH (TE $\mu\text{mol}/100\text{g}$ )	% DPPH Radical Scavenging Activity
CI1248	1178 $\pm$ 65.43b	3857 $\pm$ 213c	481 $\pm$ 24.39b	45.71
CI2230	1025 $\pm$ 10.23cd	3441 $\pm$ 322d	382 $\pm$ 14.26de	37.13
EX 116	785 $\pm$ 58.87e	3640 $\pm$ 360cd	360 $\pm$ 15.96e	34.52
EX 83	951 $\pm$ 0.54d	3667 $\pm$ 185cd	379 $\pm$ 11.59de	35.98
EX127	1403 $\pm$ 38.24a	4612 $\pm$ 333a	538 $\pm$ 1.45a	51.23
Hokuto Hadaka	1075 $\pm$ 48.44c	4433 $\pm$ 398ab	457 $\pm$ 30.27bc	42.87
Peru 16	1269 $\pm$ 69.53b	4574 $\pm$ 111ab	404 $\pm$ 39.98cde	38.61
Peru 3	828 $\pm$ 48.46e	3753 $\pm$ 402cd	399 $\pm$ 38.74de	38.03
Peru 35	1084 $\pm$ 15.87c	4217 $\pm$ 306b	421 $\pm$ 17.02cd	39.92
LSD	94.77	357.27	55.89	-

Values expressed as mean  $\pm$  standard deviation

Mean values of samples having similar letters are not significantly different ( $P < 0.05$ );

TE, Trolox Equivalent

TPC, Total Phenolic Content

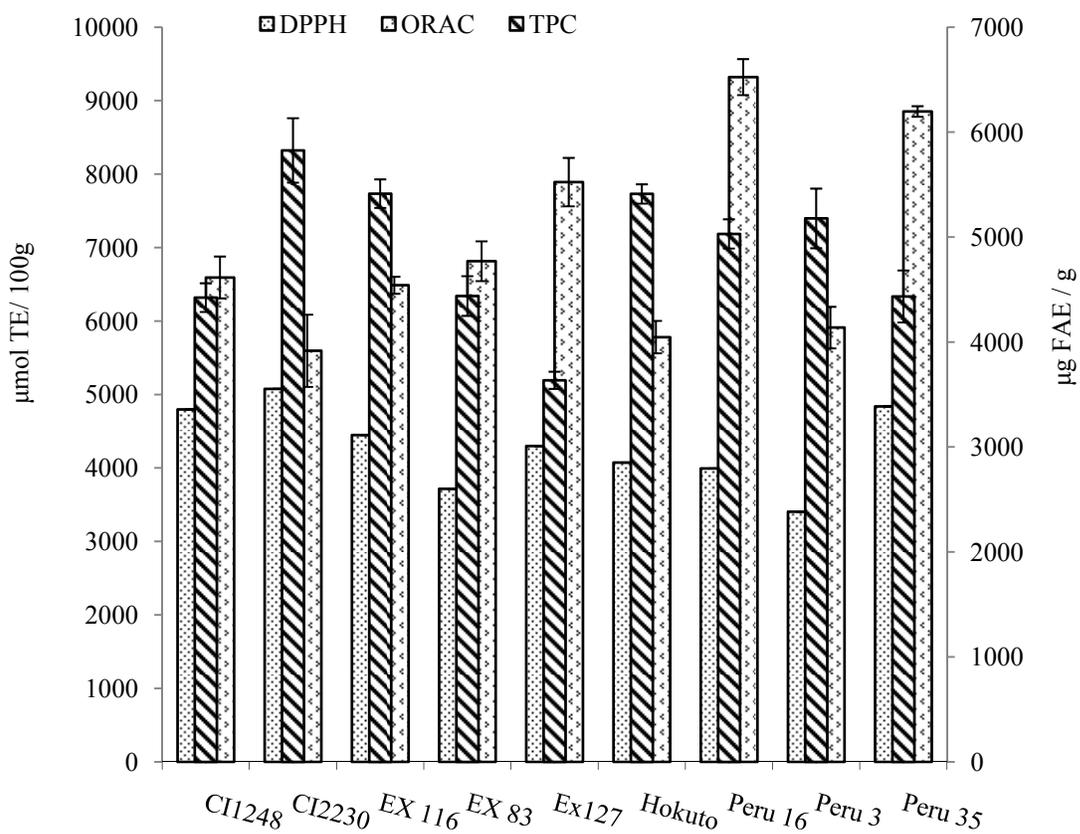
FAE, Ferulic Acid Equivalent

LSD denotes Fisher's least significant difference.

Overall, antioxidant activity measured by the ORAC assay was higher than DPPH and TPC assays and similar results were observed in a study by Tarbart et al (2009) (**Figure 2.4**). This can be explained by the difference in free radicals used in the ORAC and DPPH assays. In the former, peroxy radicals are generated by the azo compound, 2-2'-azobis (2-methyl propionamide) dichloride (AAPH). Phenolic compounds react more readily with the peroxy radical than the DPPH radical which is more stable. Furthermore, the stability of the DPPH

radical means the radical will react with more reactive phenolics substances (Stratil et al 2007). In this study, DPPH had the lowest antioxidant activities among the three antioxidant assays (ORAC, DPPH and TPC) employed as previously observed by Tarbart et al (2009). Huang et al (2005) and Karadag et al (2009) reported that antioxidants that are quick to react with peroxy radicals used in ORAC may tend to react slower with DPPH or even be inert to DPPH.

The hydrolyzed extracts exhibited the lowest antioxidant capacities and this can be attributed to the composition of the extracts. Alkaline hydrolysis targets extraction of phenolic acids (Nazck et al 2004). Furthermore, the acetone and methanol extracts may consist of more effective phenolic compounds such as flavanols hence the higher antioxidant capacities (Zhao et al 2006). PAs found in the crude acetone extract are stronger antioxidants than phenolic acids as displayed by their high antioxidant activity according to DPPH and ORAC assays (Tabart et al 2009). The acetone extract showed higher ORAC values compared to DPPH and TPC assays.



**Figure 2.4: Oxygen Radical Absorbance Capacity (ORAC) values for the aqueous methanolic and acetone extracts of different barley varieties ( $\mu\text{mol}/100\text{g TE}$ ). MET; Methanolic extract, ACE; Acetone extract.**

### 2.4.3 Total Anthocyanin Content (TAC)

Total anthocyanin content was expressed as cyanidin-3-glucoside equivalents. The TAC varied significantly among the barley genotypes ranging from 8 (Hokuto Hadaka) to 38 (CI1248)  $\mu\text{g/g}$  (**Table 2.2**). CI1248 (37.8  $\mu\text{g/g}$ ), an Israeli purple colored genotype had the highest content of anthocyanins followed by Peru 16 (30  $\mu\text{g/g}$ ). As observed during quantification of anthocyanins, the color of extracts gave an indication of the concentration of anthocyanins in the genotypes. Hokuto Hadaka (8.3  $\mu\text{g/g}$ ) and Peru 35 (13.1  $\mu\text{g/g}$ ) had relatively low TAC among the barley genotypes. Both had yellow colored extracts while CI1248, the genotype with the highest TAC had a light red extract. Acidified methanol extracts from EX116, EX83, EX127 and Peru 3 had a light pink color; however, the pink color from Ex127 was darker than the others. EX83 (14.1  $\mu\text{g/g}$ ), EX127 (19.0  $\mu\text{g/g}$ ) and Peru 3 (14.7  $\mu\text{g/g}$ ) showed intermediate TAC. No significant differences were found between TAC for EX83 and Peru 3, both yellow-colored grains.

CI1248, Peru 16, CI2230 and EX127 were the dark colored genotypes that exhibited high TAC, but Peru 35, though dark colored, showed relatively low TAC. In black barley, the pigments are located in the pericarp (Siebenhandl et al 2007). Based on the TAC, the antioxidant activity exhibited by the extracts in the methanolic extracts cannot be entirely attributed to the anthocyanins due to the low levels found in the barley genotypes. However, genotypes such as CI1248, Peru 16, CI2230 and EX127 had relatively high TAC compared to the other genotypes and also exhibited high antioxidant activities.

Wang et al (1997) determined the antioxidant capacity of 14 anthocyanins including their aglycons using ORAC assay. Cyanidin-3-glucoside had the highest ORAC activity which was 3.5 times stronger than trolox. Previous studies have also shown significant difference between TAC of sorghum cultivars (Awika et al 2004) and black rice cultivars (Ryu et al 1998). Pale yellow extracts have been reported in some white and red wheat varieties that had low anthocyanin content (Abdel-Aal et al 2006). Blue barley was reported to have TAC of 34.6 µg/g (Abdel-Aal et al 2006) which was slightly lower than the purple genotype (CI1248) that had TAC of 37.8 µg/g. Melanin-like pigment mask the pigmentation of anthocyanins (Siebenhandl et al 2007) and these may be likely the cause for the color observed in CI2230 and Peru 16, the black barley genotypes.

#### ***2.4.4 Quantification of Flavonoids in Diverse Barley Genotypes Using the Vanillin Assay***

Flavonoids are a group of phenolic compounds that exhibit antioxidant activity and have been identified in fruits, vegetables and cereals. Proanthocyanidins (condensed tannins), a subclass of flavonoids, are oligomeric and polymeric flavan-3-ols (Sun et al 1998). Flavonoids in the diverse barley genotypes were measured in the crude acetone extracts using the vanillin assay. Proanthocyanidins (PAs) were quantified as catechin equivalents (CE). The total PA content ranged from 449 to 1029 µg CE/ g barley grain dry weight basis (**Table 2.3**).

The Nepalese genotype, CI2230 (1029 µg/g) had the highest while EX83 (449 µg/g) had the lowest flavonoid contents among the barley genotypes. There were no significant differences between black hull-less genotypes, CI2230 (1029 µg/g) and Peru 35 (987 µg/g). The barley genotypes, CI2230, Peru 35 and EX116 containing elevated flavonoid contents had relatively

high antioxidant capacities as well. Although some studies have shown higher contents of PAs in hull-less compared to hulled barley (Holtekjolen et al 2006; Quinde-Axtell et al 2006), this could not be established using the nine diverse genotypes. The yellow-colored grains, EX83 (441  $\mu\text{g/g}$ ) and Peru 3 (640  $\mu\text{g/g}$ ) had significantly low PA contents. Intermediate content of PAs were found in the dark colored grains, Hokuto Hadaka (709  $\mu\text{g/g}$ ) and Peru 16 (726  $\mu\text{g/g}$ ) with no significant differences observed between them. Among the Peruvian genotypes, Peru 16 and Peru 35 displayed relatively high total PA content.

Not many researchers use the vanillin assay for quantifying PAs in barley because it is not specific for proanthocyanidins. The assay involves the reaction of an aromatic aldehyde, vanillin with a meta substituted ring of flavanols to yield a red adduct (Price et al 1978). However, most reports on quantification of proanthocyanidins are based on HPLC analysis (Dvorakova et al (2008); Friedrich et al 2002; Holtekjolen et al (2006); Quinde-Axtell et al (2006); Verardo et al (2008)). Kim reported values ranging from 15.8-131.8  $\mu\text{g CE/g}$  in the 127 colored barley lines they analyzed. According to Kim et al (2007), the blue and purple colored varieties were found to contain higher levels (83  $\mu\text{g CE/g}$ ) of PAs than the black varieties (55  $\mu\text{g CE/g}$ ). Furthermore, the concentrations of PAs in hullless barley (76  $\mu\text{g CE/g}$ ) were significantly higher than in hulled barley (56  $\mu\text{g CE/g}$ ). High flavanol contents ranging from 892 to 2000  $\mu\text{g CE/g}$  based on the vanillin assay were also reported by Dvorakova et al (2008) on 10 barley varieties grown in the same field. PA contents ranging from 449 to 1029  $\mu\text{g CE/g}$  reported in these diverse barley genotypes are higher than in lines studied by Kim et al (2007). However, CI2230 showed higher contents than 892  $\mu\text{g CE/g}$  whilst other genotypes were less than 1029  $\mu\text{g CE/g}$  but not as high as 2000  $\mu\text{g CE/g}$  reported by Dvorakova et al (2008).

#### **2.4.5 Quantification and Identification of Proanthocyanidins (PAs) using HPLC-MS**

Proanthocyanidins were quantified and identified using HPLC-MS/MS. Total PA levels determined from the nine barley genotypes were based on the 3 main compounds procyanidin B3 (pcB3), prodelphinidin B3 (pdB3) and catechin that were identified and quantified. The total PA levels ranged from 85 (EX127) – 232 (Peru 16)  $\mu\text{g CE /g}$  barley grain (dwb) (**Table 2.5**). In comparison to other literature, varying contents of total PAs have been reported by Holtekjolen et al (2006) (325-527  $\mu\text{g CE /g}$ ) and Quinde-Axtell et al (2006) (169 -395  $\mu\text{g CE /g}$ ). Trimeric proanthocyanidins were also quantified in the studies by Quinde-Axtell et al (2006) and Holtekjolen et al (2006). The differences in the flavan-3-ol contents can be attributed to genetic and environmental variation among barley genotypes and variation in extraction methods of the flavanols such as sample to solvent ratio and concentration of extraction solvent.

**Table 2.5: Quantification of Proanthocyanidins (PAs) in the Nine Barley Genotypes.**

Barley Genotype	PAs ( $\mu\text{g CE/g}$ )			
	Catechin	pdB3	pcB3	Total PA
CI1248	12 $\pm$ 0.53de	56 $\pm$ 4.17de	45 $\pm$ 1.93d	113f
CI2230	34 $\pm$ 2.23c	56 $\pm$ 7.71de	79 $\pm$ 5.75b	169c
EX 116	37 $\pm$ 3.96bc	62 $\pm$ 0.47cd	36 $\pm$ 0.13e	136e
EX 83	14 $\pm$ 1.44d	82 $\pm$ 10.38b	68 $\pm$ 2.29c	165c
EX 127	7 $\pm$ 0.65e	51 $\pm$ 2.11def	27 $\pm$ 5.76f	85h
Hokuto Hadaka	14 $\pm$ 0.43d	40 $\pm$ 5.11f	45 $\pm$ 2.67d	99g
Peru 16	45 $\pm$ 1.29a	74 $\pm$ 4.39bc	116 $\pm$ 7.34a	232a
Peru 3	40 $\pm$ 4.47ab	44 $\pm$ 1.77ef	68 $\pm$ 1.68c	151d
Peru 35	16 $\pm$ 0.14d	130 $\pm$ 11.92a	72 $\pm$ 1.43bc	218b
LSD	5.00	14.83	8.76	12.99

Values expressed as mean  $\pm$  standard deviation

PAs are expressed as  $\mu\text{g CE/g}$ . Key:pd, prodelfhinidin; pc, procyanidin

Mean values of samples having similar letters are not significantly different ( $P < 0.05$ ).

Abbreviation CE; Catechin Equivalents

LSD denotes Fisher's least significant difference.

The main flavan-3-ols identified and quantified were catechin and PA dimers namely procyanidin (B3) (27-116  $\mu\text{g CE /g}$ ), and prodelfhinidin (B3) (40-130  $\mu\text{g CE /g}$ ). Trimeric PAs with ion masses at  $m/z = 881, 865, 897$  and a catechin glucoside ( $m/z=451$ ) were also identified but were not quantified because of the very low levels that made quantification challenging. The retention times and corresponding fragments from MS/MS analysis of the identified PAs are shown in **Table 2.6** and the mass spectra are shown in **Figure 2.5**. The PAs found in the barley

genotypes have been reported by several researchers (Quinde-Axtell et al (2006), Holtekjolen et al (2006), Verardo et al (2008)).

**Table 2.6: Proanthocyanidin detected by HPLC-MS in the Nine Barley Genotypes.**

Species	Flavan-3-ol	Peak (m/z)	RT(min)	major fragments ions ( m/z)
1	(+)- Catechin C	289[M-H] <sup>-</sup>	22.02	245,221,203,177, 125,
2	Prodelphinidin B3 GC-C	593[M-H] <sup>-</sup>	13.92	407,289,177,125
3	Procyanidin B3 C-C	577[M-H] <sup>-</sup>	19.73	425, 407, 289,245,125
4	Trimer 1	897[M-H] <sup>-</sup>	12.47	711, 593, 407,289,243
5	Trimer 3/Trimer 2 C-C-C/GC-C-C	881[M-H] <sup>-</sup>	15.48	695, 577, 451,289,177
6	Trimer 3	881[M-H] <sup>-</sup>	16.41	593,407,289,177
7	Procyanidins C2/Trimer 4	865[M-H] <sup>-</sup>	20.87	577, 407, 289,243,125
8	Catechin glucoside	451[M-H] <sup>-</sup>	13.49	289, 243, 177,125
9	Barley compound 579	579[M-H] <sup>-</sup>	11.74	423, 267
10	Barley compound 468	468[M-H] <sup>-</sup>	24.69	332, 306, 289, 161, 135

Abbreviations: GC: gallocatechin subunit, C: catechin, RT: retention time

The major dimeric PAs, prodelphinidin (pdB3) and procyanidin (pcB3) together represented about 80% of the total PAs. However, pdB3 was higher than pcB3 in most of the barley genotypes. Procyanidin B3 is made up of two (+)-catechin units while prodelphinidin B3 is a dimer of (+)-catechin and (+)-gallocatechin. High contents of pdB3 were found in the black

grained Peru 35 (130  $\mu\text{g CE/g}$ ) and EX83 (82  $\mu\text{g CE/g}$ ). However, Peru 16, EX83 and EX116, all hulled genotypes, had relatively high levels of pdB3. Hokuto Hadaka had the lowest levels (40  $\mu\text{g CE/g}$ ) of pdB3. There were no significant differences in pdB3 levels among the genotypes CI2230 (56  $\mu\text{g CE/g}$ ), CI1248 (56  $\mu\text{g CE/g}$ ), EX127 (51  $\mu\text{g CE/g}$ ) and Peru 3 (44  $\mu\text{g CE/g}$ ) which had intermediate levels. Among the Peruvian genotypes Peru 3 showed lowest content of pdB3. No significant differences were observed between Peru 3, EX127 and Hokuto Hadaka. Dzorakova et al (2008) reported high contents of pdB3 in hull-less genotypes than hulled genotypes. However, this could not be established using the diverse genotypes as the hulls had been removed from four hulled genotypes prior to extractions. Studies have shown contents of pdB3 in 11 barley genotypes varying from 39 to 109  $\mu\text{g CE/g}$  (Quinde-Axtell et al 2006) and from 16 barley genotypes varying from 48 to 106  $\mu\text{g CE/g}$  (Holtekjolen et al 2006). On the other hand, contents varying from 40 to 130  $\mu\text{g CE/g}$  were displayed in this study. Peru 35 displayed contents of pdB3 greater than 110  $\mu\text{g CE/g}$  which was significantly higher than those reported earlier.

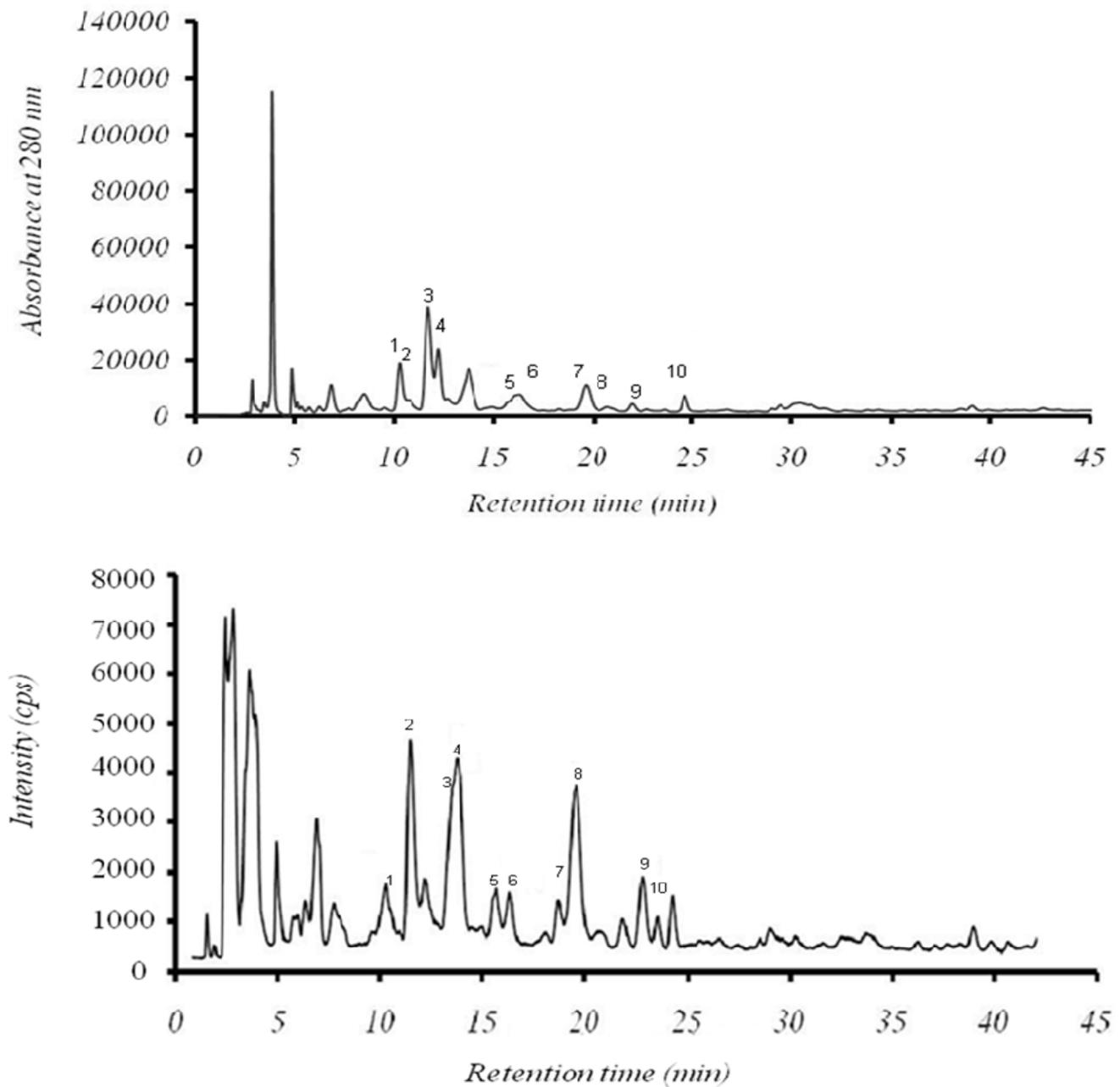
Procyanidin B3 (pcB3) was the second most abundant dimer in barley genotypes. The nine diverse barleys showed a ranged from 27 (EX127) to 116 (Peru 16)  $\mu\text{g CE/g}$ . CI2230 (79  $\mu\text{g CE/g}$ ), the Nepalese genotype had the second highest content of pcB3 that was not significantly different from Peru 35. The high contents of pcB3 and pdB3 in CI2230 may be partially responsible for the high antioxidant activity exhibited by this genotype. No significant differences were found between Peru 3 (68  $\mu\text{g CE/g}$ ) and Peru 35 (72  $\mu\text{g CE/g}$ ) in pcB3 levels both of which had intermediate contents. EX127, a cross between Canadian and Germany genotype, had low levels of the individually quantified flavan-3-ols. The cross also displayed

relatively low total phenolic contents. While contents of pcB3 varying from 27 to 116  $\mu\text{g CE/g}$  were obtained in this study, other reports show contents varying from 17 to 67  $\mu\text{g CE/g}$  (Dvorakova et al 2008), 63 to 126  $\mu\text{g CE/g}$  (Holtekjolen et al 2006) and 40 to 99  $\mu\text{g CE/g}$  (Quinde-Axtell et al 2006) for pcB3. Peru 16 showed levels of pcB3 higher than 99  $\mu\text{g CE/g}$  reported in literature. CI2230, Peru 35 and EX83 displayed contents of pcB3 higher than 67  $\mu\text{g CE/g}$  while the rest of the genotypes were within range.

Catechin was a minor monomeric flavanol constituent in all the genotypes (**Table 2.5**). Catechin ( $m/z=289$ ) had contents varying from 7 to 45  $\mu\text{g CE/g}$  and accounted for 8 to 27 % of the total PAs quantified in the diverse barley genotypes. Peru 16 exhibited the highest content; however, no significant differences were observed between Peru 16 and Peru 3. The levels of catechin in EX116 (37  $\mu\text{g CE/g}$ ) was relatively high. There were no significant differences in the catechin levels among the barley genotypes Peru 35 (16  $\mu\text{g CE/g}$ ), Hokuto Hadaka (14  $\mu\text{g CE/g}$ ), EX83 (14  $\mu\text{g CE/g}$ ) and CI1248 (12  $\mu\text{g CE/g}$ ). EX127 (7  $\mu\text{g CE/g}$ ) displayed the lowest quantities of catechin. While the hulled genotypes, Peru 16, Peru 3 and EX116 had relatively high contents of catechin, there was no distinct relationship pertaining to catechin contents and type of caryopsis. Quinde-Axtell et al 2006 found higher contents of catechin in 4 hull-less genotypes compared to 7 hulled genotypes. High contents of catechin ranging from 15 to 57  $\mu\text{g CE/g}$  were also reported by Dvorakova et al (2008).

Flavanol glycosides are naturally occurring in plants and appear as 3-, 5-, 7- *O* glycosides and as 6- and 8-*C* glucosides (Harborne et al 1999). Catechin glucoside with an ion  $[\text{M-H}]^-$  at  $m/z$  451 was identified and co-eluted at similar retention time as the dimer, pdB3. Catechin

glucoside was highest in Peru 35, EX83 and Peru 3 and made up about 25% of pdB3 peak area. Lower levels of catechin glucoside approximately less than 10% were observed in the other genotypes. Catechin glucoside has also been detected and described by Dvorakova et al (2008) in 10 barley varieties. Friedrich et al (2002) isolated the catechin glucoside from 20 barley varieties and found quantities ranging from 6 - 38  $\mu\text{g CE/g}$ , values similar to those found in the present investigation. Other major peaks detected in the nine barley genotypes were m/z 579, m/z 741 and m/z 468 but their identity was not established (**Table 2.6**).

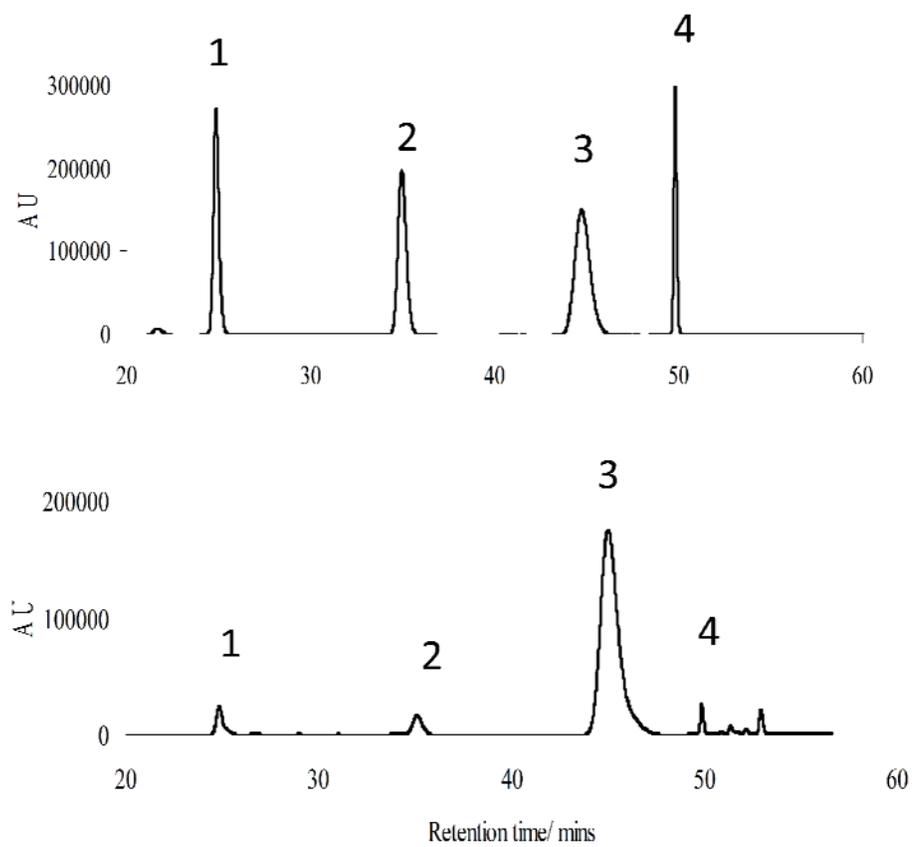


**Figure 2.5: HPLC-UV-ESI-MS profile for barley genotype Peru 35 (a) UV chromatogram (280nm), (b) Total Ion (full scan) chromatogram (TIC), for the following; peak 1= barley compound 579, peak 2= trimer 1 ( $m/z = 897$ ), peak 3= catechin glucoside, peak 4 = prodelphinidin B3, peak 5 = trimer 3/trimer 2( $m/z=881$ ), peak 6 = trimer 3, peak 7 = procyanidin B3, peak 8 = trimer 4 ( $m/z= 865$ ), peak 9 = catechin, peak 10= barley compound 468.**

#### 2.4.6 Quantification of Phenolic acids In Diverse Barley Genotypes

Alkaline hydrolysis was employed for the release of bound phenolics. Phenolic acids that were identified and quantified in the diverse food barley genotypes were the hydroxycinnamic acid derivatives *p*-coumaric, ferulic, sinapic and caffeic acids and the hydroxybenzoic acid derivative, vanillic acid (Figure 2.6). Their corresponding quantities in each barley genotypes are shown in

**Table 2.7.** Using HPLC-ESI-MS analysis for phenolic acids, the 8-5' benzofuran form (m/z 341), 5-5'-DFA and 8-*O*-4'-DFA (m/z 341) were detected (Figure 2.7); however, quantification was done for the 8-*O*-4'-DFA and 8-5' benzofuran form. 5-5'-DFA was detected in too small amounts for quantification. Total phenolic acids in the nine genotypes ranged from 316 µg/g for Peru 3 to 579 µg/g for Peru 35. Barley genotypes with high ferulic acid content also had high levels of the ferulic acid dehydrodimers. Peru 35, EX127, CI2230, CI1248 had relatively high contents of ferulic acid and ferulic acid dehydrodimers while Peru 3 , EX116 and EX83 had relatively low contents.



**Figure 2.6: HPLC chromatograms at 320nm of (A) standards and (B) barley genotype EX127 after alkaline hydrolysis: peak 1-caffeic acid, peak 2-p-coumaric acid, peak 3-ferulic acid, peak 4-sinapic acid.**

Ferulic acid (3-methoxy-4-hydroxycinnamic acid) (FA) was the most abundant phenolic acid with contents ranging from 246 to 485  $\mu\text{g/g}$  for the barley genotypes. FA made up 88-94% of the total phenolic acids. Highest FA concentrations were found in the black grain, Peru 35. The lowest levels were observed in Peru 3, however, there were no significant differences between yellow grained, EX116 (283  $\mu\text{g/g}$ ) and Peru 3 (246  $\mu\text{g/g}$ ). Generally, Peru 3 tended to exhibit inferior antioxidant characteristics compared to the other Peruvian genotypes, Peru 35 and Peru 16. The dark colored grains, Hokuto Hadaka (416  $\mu\text{g/g}$ ) and Peru 16 (392  $\mu\text{g/g}$ ) had relatively high FA contents which were not significantly different. High FA contents of 338 and 335  $\mu\text{g/g}$  were also observed in the dark colored Nepalese (CI2230) and Israeli (CI1248) genotypes respectively. Dark colored grains, CI2230, CI1248, Peru 16 and Peru 35 displayed similar contents of phenolic acids.

Using alkali hydrolysis, Quinde-Axtell et al (2006) reported FA contents ranging from 301 to 567  $\mu\text{g/g}$  in 11 barley genotypes. Holtekjolen et al (2006) also reported contents ranging from 403 to 723  $\mu\text{g/g}$  of FA in 16 barley varieties of diverse origin; however, values ranging from 452 to 605  $\mu\text{g/g}$  were observed after an acid hydrolysis of 18 feed and malting barleys (Zupfer et al 1998). The barley genotypes used in this study showed contents ranging from 246 to 485  $\mu\text{g/g}$ . Peru 3 and EX116 had levels of FA lower than 301  $\mu\text{g/g}$  found in literature while the rest of the genotypes showed levels greater than 301  $\mu\text{g/g}$  which is within range. FA has been reported as the most abundant phenolic acid in wheat grains as well (Klepacka et al 2006). In other cereals, contents of FA ranging from 784 to 1980  $\mu\text{g/g}$  in durum wheat (Lempereur et al 1998) and 859 to 1174  $\mu\text{g/g}$  in rye (Andreasen et al 2000) have been observed using alkaline hydrolysis. In six wheat genotypes analyzed by Mpofu et al 2006, FA accounted for 50-65% of

the amount of phenolic acids ranging from 371 to 441  $\mu\text{g/g}$ . The barley genotypes had relatively low ferulic acid content compared to other cereals. Apart from genetic and environmental variation, differences in phenolic acid contents can be attributed to the differences in solvents concentrations and time of extraction employed. For instance, Quinde-Axtell et al (2006) used a combination of acid and enzyme hydrolysis while Holtekjolen et al (2006) used alkali hydrolysis for extraction of phenolic acids. Moreover, Hernanz et al (2001) and Holtekjolen et al (2006) employed 2M NaOH while 4M NaOH was used for this study for the alkali hydrolysis. Hydrolysis procedures have an effect on the phenolic acids detected (Ross et al 2009).

*p*-Coumaric acid (*p*-hydroxycinnamic acid), the second most abundant phenolic acid showed contents ranging from 5 to 90  $\mu\text{g/g}$ . It accounted for 1-18% of total phenolic acids in the nine barley genotypes. Peru 16 had the highest content of *p*-coumaric acid (90  $\mu\text{g/g}$ ) while CI2230 had the lowest content of 5  $\mu\text{g/g}$ . All the hulled genotypes, Peru 3 (12  $\mu\text{g/g}$ ), EX83 (11  $\mu\text{g/g}$ ) and EX116 (10  $\mu\text{g/g}$ ) had significantly high levels of *p*-coumaric acid. However, Peru 35 (13  $\mu\text{g/g}$ ), a hull-less genotype displayed comparatively high contents of *p*-coumaric acid. The content of *p*-coumaric acid was significantly low in hull-less genotypes (CI2230 and CI1248). Studies have reported that *p*-coumaric acid is concentrated in the hull (Holtekjolen et al 2006, Nordkvist et al 1984, Hernanz et al 2001), however hulls were removed prior to analysis of barley genotypes. Holtekjolen et al (2006) found contents of *p*-coumaric acid ranging from 15 to 374  $\mu\text{g/g}$  in 16 barley genotypes while Quinde-Axtell et al (2006) reported quantities of *p*-coumaric acid that ranged from 4 to 68  $\mu\text{g/g}$  in 11 barley genotypes depending on presence or absence of hulls.

Substantial amounts of caffeic acid were detected in the nine barley genotypes ranging from 4 to 15  $\mu\text{g/g}$ . The highest content of caffeic was found in the black barley, CI2230 while EX116 had the lowest. Peru 35 (6  $\mu\text{g/g}$ ), which had the highest concentration of FA, was also observed to have the second lowest concentration of caffeic acid. Peru 3 (11  $\mu\text{g/g}$ ) and Peru 16 (14  $\mu\text{g/g}$ ) had relatively high caffeic acid contents. Intermediate levels of caffeic acid were found in EX 83 (9  $\mu\text{g/g}$ ). The purple colored grains, EX127 (11  $\mu\text{g/g}$ ) and CI1248 (13  $\mu\text{g/g}$ ) showed no significant difference between them. Caffeic acid accounted for about 1 to 3 % of the total phenolic acids that were quantified in the nine diverse barley genotypes. Losses of phenolic acids have been reported in literature. For instance, in the determination of phenolic acid levels in rapeseed using 4N NaOH for 4 hours under nitrogen, a loss of 10% was estimated for cinnamic acid derivatives such ferulic and p-coumaric acid. However, a loss of 67 and 37 % was reported for caffeic and sinapic acid respectively (Krygier et al 1982). Similar potential losses are anticipated in the barleys as the same alkaline hydrolysis was employed for 4 hours. Recovery of phenolic acids can be aided by the addition of ascorbic acid and ethylenediaminetetraacetic acid (EDTA) (Nardini et al 2002). Caffeic acid that was detected in the barley genotypes, has been reported to possess antitumor effects against colon cancer (Oltholof et al 2001). Hernaz et al (2001) found contents of caffeic acid ranging from 7 to 18  $\mu\text{g/g}$  using 11 barley genotypes of malting and feed quality. Quinde-Axtell et al (2006) also reported contents ranging from 15 to 36  $\mu\text{g/g}$  in 11 barley genotypes. Caffeic acid contents in the barley genotypes were in the same range as those reported in literature.

**Table 2.7: Contents of Individual Phenolic Acids in the Nine Whole Barley Genotypes**

Barley Genotype	µg/g of dry barley								
	Ferulic	<i>p</i> -Coumaric	Caffeic	Sinapic	Vanillic	8-O-4' DFA	8,5' DFA benzofuran form	Total DFAs	Total phenolic acids
CI1248	335±0.56c	7±0.37d	11±0.69bc	9±0.41d	9±0.55b	19±0.05c	16±1.04bcd	35	406
CI2230	338±4.19c	5±0.37e	15±0.29a	35±1.28a	9±0.33cb	23±0.24bc	16±0.38bcd	39	441
EX 116	283±8.61de	10±0.12c	4±0.1g	6±0.79ef	8±0.82cd	13±1.70d	10±0.74d	23	334
EX 83	323±5.42cd	11±0.76c	9±0.03de	5±0.20f	7±0.06d	13±0.60d	11±0.40d	24	379
EX127	464±36.16a	12±0.80cb	13±0.89ab	16±1.51b	8±0.66cb	20±0.14c	25±1.15a	45	558
Hokuto	416±34.27b	7±0.90d	10±2.68ef	10±1.44c	8±0.67cd	24±0.68b	16±0.94ab	40	491
Hadaka									
Peru 16	392±5.28b	90±0.02a	14±0.60a	9±0.37d	11±0.19a	15±0.01d	11±0.82cd	26	541
Peru 3	246±1.34e	12±0.82b	11±0.69cd	8±0.25de	6±0.44e	13±0.52d	11±0.32d	24	316
Peru 35	485±32.39a	13±0.70b	6±2.45f	7±1.15d	9±0.24cb	34±4.18a	24±3.69abc	58	579
LSD	45.38	1.31	2.13	1.77	1.41	3.73	7.05	-	-

Each value is the mean± standard deviation of duplicate determination

Mean values of samples having similar letters are not significantly different (P<0.05).

LSD denotes Fisher's least significant difference.

The sinapic acid content ranged from 5 to 35  $\mu\text{g/g}$ . The black barley, CI2230 exhibited the highest contents of sinapic acid as well as caffeic acid. EX 83 (5  $\mu\text{g/g}$ ), exhibited the lowest sinapic acid content while EX127 (16  $\mu\text{g/g}$ ) had intermediate contents. No significant differences were observed among the Peruvian genotypes, Peru 16 (9  $\mu\text{g/g}$ ), Peru 3 (8  $\mu\text{g/g}$ ) and Peru 35 (7  $\mu\text{g/g}$ ) and CI1248 (9  $\mu\text{g/g}$ ) in sinapic acid contents. Sinapic acid accounted for about 1 to 9 % of the total phenolic acids that were quantified in the diverse barleys. There were no significant differences in the sinapic acid content among the dark colored barleys (CI1248, Peru 16, and Peru 35) as observed with FA contents. There is limited literature on sinapic acid contents of barley. In the study by Hernaz et al (2001), sinapic acid was not detected. However, alkaline hydrolysis released esterified phenolic acids leading to the detection of sinapic acid and other acids in two Canadian barley cultivars. (Madhujith et al 2006).

Vanillic acid was the major hydroxybenzoic acid derivative that was detected and quantified in the barley genotypes. Quantification of vanillic acid was carried out at 291 nm while that of the other phenolic acids was measured at 320 nm. Vanillic acid was found to have high absorption at 291 nm than at 320 nm. Contents of vanillic acid ranged from 6 to 11  $\mu\text{g/g}$ . The highest contents of vanillic acid were found in Peru 16 (11  $\mu\text{g/g}$ ) while Peru 3 (6  $\mu\text{g/g}$ ) exhibited the lowest. CI2230 and CI1248 (9  $\mu\text{g/g}$ ) exhibited intermediate contents with no significant differences between them.

In plant materials, the main ferulic acid dehydrodimers identified include 8-O-4'-DFA, 8-5' DFA benzofuran, 8-5' DFA and 5-5' DFA forms with 8-O-4'-DFA being the most prominent (Andreasean et al 2000). Ferulic acid dehydrodimers were quantified using the response factors of the trans-cinnamic acid at 280 nm. DFAs identified and quantified in the diverse barleys were 8-O-4' DFA and 8-5' DFA benzofuran form. Their structures are shown in **Figure 2.7**. Total content of the ferulic acid dehydrodimers 8-O-4' DFA and 8-5' DFA, varied between 23-58 µg/g in the diverse barleys (

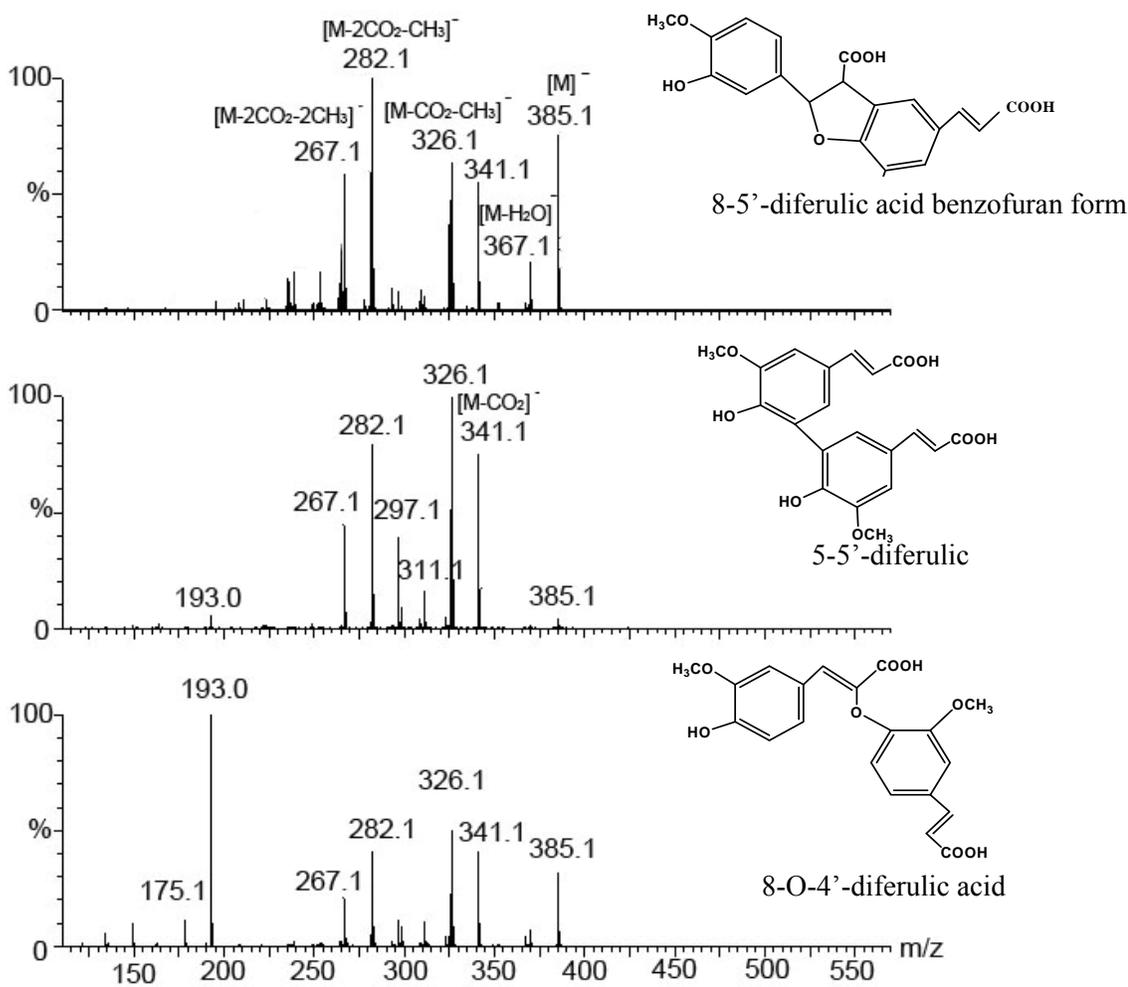
**Table 2.7**). 8-O-4' DFA was the most abundant dimer with contents ranging from 13 to 34 µg/g. Peru 35 (34 µg/g) had the highest amounts of 8-O-4' DFA while EX116, Peru 3 and EX83 (13 µg/g) had the least. 8-5' DFA benzofuran form was identified in quantities ranging from 10 to 25 µg/g (

**Table 2.7**). EX127 and Peru 35, both dark colored, exhibited high and similar levels of 8-5' DFA while EX116, a yellow-colored grain, had the lowest content. Peru 35 had the highest levels of 8-5' DFA and 8-O-4' DFA. Dimers, 8-5' DFA and 8-O-4' DFA, were detected in higher quantities higher than the monomeric phenolic acids, *p*-coumaric, caffeic, sinapic and vanillic. Ferulic acid dehydrodimers in barley grains have also been reported in varying contents from 73.5 to 118 µg/g dry weight and 22.6 to 45 µg/g dry weight for 8-O-4' and 8-5' DFA respectively in 11 barley varieties of malting and feed quality (Hernaz et al 2001). Holtekjolek et al (2006) found quantities ranging from 40 to 90 µg/g and 68 to 114 µg/g for 8-O-4' and 8-5' DFA respectively in 16 barleys after alkaline hydrolysis. Low contents of DFAs were displayed in the diverse barleys compared to literature. DFAs vary depending on cereal type, varieties in

one cereal and differences in dietary fibre fractions such as soluble or insoluble (Bily et al 2004; Bunzel et al 2001; Renger et al 2000). Furthermore, method of extraction may cause variation in DFA levels. Andreasen et al (2000) used enzyme hydrolysis coupled with alkaline hydrolysis in their extraction of DFA in rye. To prevent oxidation, hydrolysis was carried out under nitrogen using a magnetic stirrer in this study. However, Holtekjolen et al (2006) did not prevent oxidation in their assay.

The radical scavenging activity of phenolic acids is highly dependent on their structure i.e. the number of hydroxyl groups on the benzene ring and the ortho substitution with electron donor methoxy group which increases the stability of the phenoxy radical (Rice-Evans et al, 1996). Furthermore, *o*- or *p*- dihydroxylic groups that contain one free and one alkylated hydroxyl group (usually methoxyl) are stronger antioxidants. Based on structure, among the cinnamic acid derivatives, caffeic acid is a stronger antioxidant than ferulic acid, which is a more effective antioxidant, compared to *p*-coumaric acid (Holtekjolen et al 2006). Kikuzaki et al 2002, found that the radical scavenging activity on DPPH<sup>•</sup> were in the decreasing order caffeic > sinapic > ferulic > *p*-coumaric. A similar trend was also reported by Cuvelier et al 1992, when they evaluated the antioxidant capacity of the caffeic, sinapic, ferulic and *p*-coumaric in a methyl linoleate system. Introduction of a second hydroxyl group on the ortho or para position increases antioxidant activity hence caffeic acid is a more efficient antioxidant in vitro when compared to the monophenol, *p*-coumaric (Holtekjolen et al 2006). The distribution of caffeic, sinapic, ferulic and *p*-coumaric acids in the barley genotypes may help explain the variation in antioxidant activity of the hydrolyzed extracts. The high antioxidant capacity of purple grained, EX127, can be attributed to the presence of high ferulic acid contents and the relatively high

caffeic acid contents. CI2230, had high contents of caffeic and sinapic acid which are very strong antioxidants according to Kikuzaki et al (2002). Though Peru 3 had the second highest contents of *p*-coumaric acid, it generally had low contents of caffeic, sinapic and ferulic acids and this was the likely the cause for the low antioxidant capacity that was observed.



**Figure 2.7:** MS spectra obtained from peaks after HPLC-PAD-MS-MS analysis with negative electrospray ionization for EX127 and the respective structures of the isomers of

**ferulic acid dehydrodimers of corresponding molecular mass 385. The Y axis is the relative abundance and the x-axis shows the m/z (mass to charge ratio).**

#### **2.4.7 Correlations Analysis**

Correlation values generated in this study are based on limited diverse barley genotypes. The highest correlation coefficient was found between the total PA content and the DPPH assay ( $r=0.82$ ,  $n=9$ ,  $p=0.007$ ) for the acetone extracts. The total PA levels ranged from 85 (EX127) – 232 (Peru 16)  $\mu\text{g/g}$  CE of dry weight of barley whole grain (**Table 2.5**). The positive correlation shows that the flavan-3-ols most likely work best by donating electrons and thereby inhibiting free radical reactions. The DPPH radical scavenging activity was likely due to the presence of PAs. PAs were the major phenolic compounds found in the nine barley genotypes of diverse origin. PdB3 and catechin have been reported to be the main contributors to the antioxidant activity of barley (Dvořáková et al 2008). There was no correlation between ORAC and DPPH ( $r=0.04$ ,  $n=9$ ,  $p=0.9$ ) for the acetone extracts of the barley genotypes. In the ORAC assay the peroxy radicals are generated by the azo compound AAPH and these radicals react readily unlike the stable nitrogen radical, DPPH. Tabart et al (2009) found no correlation between either trolox equivalent antioxidant capacity (TEAC) or the DPPH and the ORAC data for flavanols, anthocyanins, flavanones, flavan-3-ol and phenolic acids.

The alkaline hydrolyzed extracts, the TPC for the alkaline hydrolyzed extracts had fairly strong positive correlations with both DPPH ( $r=0.774$ ,  $p=0.01$ ) and ORAC ( $r=0.772$ ,  $p=0.01$ ). These results show that the phenolic acids were the major compounds responsible for the antioxidant capacities in these alkaline hydrolyzed extracts. A positive correlation between TPC

and the total amount of phenolic acids based on HPLC analysis ( $r=0.83$ ,  $p=0.008$ ) was also observed. The L (degree of lightness) and  $b^*$ (degree of yellowness) values for the grains had a high positive correlation ( $r=0.869$ ,  $p=0.02$ ) indicating a relation between degree of lightness and grain color. There was strong correlation between degree of lightness ( $L^*$ ) and total anthocyanin content (TAC) and total flavonoid content as measured by the vanillin assay. The correlation value between degree of lightness (L) and TAC was 0.956 ( $p= <0.0001$ ) while the correlation value between degree of lightness (L) and total flavonoid content was 0.90 ( $p=0.0008$ ) for the nine barley genotypes thereby indicating a relationship between grain color and anthocyanin / flavonoid.

## **2.5 Conclusion**

The nine genotypes reflected the diversity in phenolic compounds that can be found in barley grains. The phenolic acids, PAs and anthocyanins identified and quantified had significant contribution to the overall antioxidant capacity of the barley whole grain. Dark colored grains such as CI2230, Peru 35, Ex127 and Peru 16 tended to displayed higher contents of proanthocyanidins, phenolic acids and anthocyanins. The present study determined the antioxidant capacities of the nine barley extracts in both methanolic and acetone extracts, highlighting the selectiveness of aqueous acetone for barley phenolic compounds. Presence of phytochemicals in CI2230, Peru 35, Ex127 and Peru 16 opens avenues for better opportunities for the use of barley in the food products.

## ***Chapter 3. Effects of Sprouting on the Antioxidant Activity and Phenolic Acid Content of Food Barleys of Diverse Origin***

### ***3.1 Abstract***

Cereal sprouts are believed to be nutritionally rich compared to cereal grains. Barley hull-less genotypes of diverse origin were partially sprouted in an automatic sprouter for 19 hours. Four hull-less barley genotypes studied included CI2230 from Nepal, CI1248 from Israel, Peru 35 from Peru and EX127, a cross between Canadian and German genotypes. The amounts and composition of free, conjugated, bound and total phenolic acids were determined in raw and partially sprouted hull-less whole genotypes grown on the same site in 2007. The concentration of the phenolic acids in the different phenolic acid fractions were in the order bound > conjugated > free, with bound making up around 81-98% of the total phenolic acid concentration, soluble conjugated constituting between 4 and 18% and free phenolic acid constituting between 0.07 and 0.2%. Ferulic acid was the only free phenolic acid quantified with quantities ranging from 1.59 to 3.69  $\mu\text{g/g}$  in partially sprouted and their controls. The soluble conjugated acids identified and quantified using HPLC-MS analysis included ferulic, p-coumaric, caffeic, sinapic and vanillic acid. The DPPH radical scavenging capacity and total phenolic content was also determined for the crude methanolic and acetone extracts for both raw grain and partially sprouted barleys. Raw grains exhibited higher phenolic content and antioxidant activity in both extracts. Partial sprouting was observed to significantly increase the soluble conjugated phenolics.

### 3.2 Introduction

Cereal grains are a good source of phenolic acids which occur in free, conjugated and bound form. Phenolic acids are the major phenylpropanoid compounds and secondary plant metabolites found in cereals. The most abundant cinnamic acid derivative is ferulic acid (FA) followed by *p*-coumaric acid (*p*-CA) (Holtekjolen et al 2006). Phenolic acids exist in two classes namely hydroxybenzoic acids and hydroxycinnamic acids. Examples of the former include gallic, *p*-hydroxybenzoic, vanillic, syringic and protocatechuic acids. The hydroxycinnamic acids include *p*-coumaric, caffeic, ferulic and sinapic acids these are commonly found in barley grain (Von Wettstein 1985). The hydroxycinnamates; caffeic, ferulic and sinapic are formed through the hydroxylation and methylation of *p*-coumaric in the position 3 and 5 (Shahidi et al 2004).

Determination of phenolics acids is affected by extraction and hydrolysis conditions. Numerous extraction procedures have been employed in literature (Robbins 2003). Soluble phenolic can be extracted using organic solvents such as methanol acetone and or water (Mattila et al 2005). Insoluble bound and soluble conjugated phenolic acids have liberated through alkaline, acid and enzyme or both. Quantification of phenolic can be achieved through thin-layer chromatography (Schmidtlein et al 1975), gas-liquid chromatography, gas chromatography-mass spectroscopy (Wu et al 1999) or capillary electrophoretic methods (Fernandes et al 1996). However, high performance liquid chromatography (HPLC) is currently the most widely used quantification technique (Hernaz et al 2001, Holtekjolen et al 2006, Zupfer et al 1998).

Compared to cereal grains and their products, cereal sprouts are believed to have a greater nutritive value (Price 1988). The sprouting process can be semi or fully automated. Sprouting is applied to barley as part of the malting process and results in modification of the grain. Since most of the phenolic acids found in barley occur in bound form, sprouting releases some of these phenolic acids through the activation of enzymes that act on cell wall polymers such as endo-(1→3), (1→4) β-glucanase, endo-(1→3) - β-glucanase, endo-(1→4) β-xylanase, endopeptidase, carboxypeptidase, α-amylase (types I, II, III), β-amylase, α-glucosidase, lipase, phospholipase and limit dextrin (Newman et al 2008). Sprouting of cereals also results in the softening of the grain thereby resulting in easier incorporation into other foods and also contribute to the organoleptic properties of food (Mwikya 2001). Sprouted barley has potential as a food additive. This study was aimed at determining free, soluble conjugated and insoluble bound phenolic acid composition and antioxidant activity of partially sprouted whole barley grains of diverse origin.

### ***3.3 Materials and Methods***

#### ***3.3.1 Sample description***

Four hull-less barley genotypes namely, Peru 35, EX127, CI1248 and CI2230 were studied. The samples are of diverse geographical origin and age. Peru 35 is a food barley from Peru that originated from an international centre for barley germplasm development (CYMMIT) in Mexico. CI1248 and CI2230 are landraces collected from Israel in 1972 and Nepal in 1969 respectively. EX127 is a cross between a Brandon six-row feed line and the 19th century barley variety Faust, from Germany. Faust is a 'hooded lemma' variety that was used for grazing.

These samples were grown in 2007 at the Agriculture and Agri-Food Canada's Brandon Research Centre located within the Barley Breeding and Genetics program, Brandon, Manitoba. All the genotypes are 6-rowed except for CI2230 which is 2-rowed. These diverse barleys were further classified according to their color based on visual appearance. The black grained varieties included Peru 35 and CI2230 while EX127 and CI1248 showed a characteristic purple colour.

### **3.3.2 Chemicals**

2, 2-Diphenyl-1-picrylhydrazyl (DPPH<sup>•</sup>), Folin-Ciocalteu reagent and phenolic acid standards were obtained from Sigma- Aldrich (St Louis, MO). Phenolic acid standards used for HPLC analysis include ferulic, caffeic, *p*-coumaric and sinapic acid. Trolox was purchased from Fisher Acros Organics (Fair Lawn, New Jersey) for the use as a standard for DPPH radical scavenging activity. The solvents acetone, ethyl acetate, methanol, acetonitrile, acetic acid, hydrochloric acid were HPLC grade and obtained from Fisher Scientific, Fair Lawn, New Jersey. The sodium hydroxide and sodium carbonate was purchased from Fisher (Fair Lawn, New Jersey).

### **3.3.3 Sprouting of Barley Sample**

Four barley genotypes (25 g each) were sprouted in duplicates for 19 hours by using a FreshLife Automatic Sprouter, Model 2000 (Tribest Corp., Santa Fe Springs, CA). The automatic sprouter had a designed pump with a temperature sensor. Sprouting was conducted at room temperature. The grains were kept moist by sprinkling water once every hour through the built-in automated sprinkler. Sodium hypochlorite (200ppm) was added to the sprinkling water and served as an antimicrobial agent. Sprouting was arrested at the sign of shoots appearing but

before they started elongating. The sprouts had moisture content ranging from 44 to 49%. All samples were freeze-dried (Unitop 600L Freeze Mobile 6; VirTis, Gardiner, NY), stored at -40 °C and ground prior to analysis. The difference between the dry matter content of raw grain and that of partially sprouted grain was considered as loss of dry matter.

### **3.3.4 Extractions**

*Acetone Extracts* Acetone extracts were prepared according to a method by Naczki et al (2004) as modified by Hosseinian et al (2007). Briefly, ground barley (1 g) was mixed with 20 mL of acetone, water and acetic acid (70:29:1, v/v) and sonicated for 30 mins (Branson-3200R-2 sonicator, Shelton, CT). Subsequently the reaction mixture was centrifuged for 15 mins at 4000 rpm (Centrifuge CL31 multispeed, Thermo Electron Industries SAS, France). The supernatant was retained and used as crude extract for DPPH radical scavenging activity and total phenolic content (TPC). Extractions were done in duplicates for sprouted barleys and their controls.

*Methanolic Extracts* Extracts were prepared according to Naczki et al 2004 as modified by Hosseinian et al (2008) and Bellido & Beta (2009) with some further modification. Ground barley (1 g) was suspended in 10 ml of acidified methanol (1N HCL, 85:15, v/v) and its pH adjusted to 1.0 using 1N HCl. The suspension was shaken for 2.5 h at room temperature (25°C) using a rotary shaker (Fermentation Design Inc, Allentown, PA) at 250 rpm and centrifuged at 40000 rpm for 15 min using a multispeed CL31 centrifuge (Thermo Electron Industries SAS, France). The supernatant was retained at -40°C and used for DPPH radical scavenging activity and total phenolic content (TPC). Extractions were done in duplicates for sprouted barleys and their controls.

### **3.3.5 Measurement of Total Phenolic Content**

The total phenolic content of barley extracts was determined according to a laboratory procedure using Folin-Ciocalteu method (Singleton and Rossi 1965). Briefly, a barley extract (200 $\mu$ L) was added to 1.5 mL of 10 fold diluted and freshly diluted Folin-Ciocalteu reagent freshly prepared and equilibrated for 5 minutes after vortexing. Subsequently, 1.5 mL of sodium carbonate (60 g/L) was then added to neutralise the mixture and vortexed. After 90 mins of reaction at ambient temperature, absorbance was measured at 725 nm against reagent blank and used to calculate the total phenolic content using ferulic acid as a standard. Concentrations ranging from 100-1000  $\mu$ M of ferulic acid were used for quantification. Analyses were conducted in triplicates. The results were expressed as ferulic acid equivalents on a dry matter basis.

### **3.3.6 Determination of DPPH radical Scavenging Activity**

The DPPH method of Brand- William et al (1995) as modified by Li et al (2005) was further modified for this assay. Briefly, a 60 $\mu$ M DPPH radical solution was freshly prepared from a stock solution of 300  $\mu$ M. Barley extracts (100  $\mu$ L) were reacted with 3.9 mL of the DPPH radical solution for 60mins. The absorbance at 515nm was measured against a blank of pure methanol at t = 0, 5, 10, 20, 30, 40, 50 and 60 mins and used to estimate the level of free radical scavenging ability. Concentrations ranging from 100 to 1000  $\mu$ M were used for the Trolox standard curve. Antioxidant activity was calculated as a percentage DPPH radical scavenging activity expressed using Trolox equivalents (TE) per gram of dry matter (DM) using the following equation;

$$\% \text{ DPPH} \cdot \text{ scavenging activity} = \left(1 - \frac{A_{\text{sample},t}}{A_{\text{control},t}}\right) \times 100$$

where  $A_{\text{sample}, t}$  is the absorbance of the sample at time = 30 mins and  $A_{\text{control}, t}$  is the absorbance of the control at time = 0 mins.

### **3.3.7 Extraction of Phenolic Acids**

*Free* Free phenolic acids were extracted from barley grains by blending 5 g of ground barley flour with 25 mL of 80% methanol. After 30 mins of sonication, the mixture was centrifuged for 10 mins. The supernatant was removed and the extraction was repeated with the residue. Supernatants from the two extractions were combined and concentrated in a rotatory evaporator. The extract was reconstituted in 5 mL of 80% methanol. The reconstituted extract (400  $\mu$ L) was used for HPLC analysis of free phenolic acids while the remaining extract was used for further extractions.

*Soluble conjugated* Extracts from the free phenolic extraction above were used for the soluble conjugate phenolic acids. The extract (4.6 mL) was hydrolysed with 50 mL of 4 M NaOH on a stirring plate under nitrogen gas for 4 h. After digestion, the solution was adjusted to a pH 1.5~2.0 with 6 M HCl and then extracted with 70 mL of ethyl acetate three times. The combined ethyl acetate fractions were evaporated to dryness and reconstituted in 3 mL of 50 % methanol. Prior to HPLC analysis, they were filtered through a 0.45  $\mu$ m syringe filter.

*Insoluble bound* The residue remaining from the extraction of free phenolic acids was hydrolyzed with 60 mL of 4 M NaOH on a stirring plate under nitrogen gas for 4 h. After digestion, the solution was adjusted to a pH 1.5~2.0 with 6 M HCl and centrifuged for 1 hour at

5000 rpm (Sorvall RC5C, Sorvall Instruments, DuPont, Wilmington, DE). Subsequently the supernatant was extracted with 70 mL of ethyl acetate three times. The combined ethyl acetate fractions were evaporated to dryness and reconstituted in 3 mL of 50 % methanol. Prior to HPLC analysis, they were filtered through a 0.45  $\mu\text{m}$  syringe filter.

### **3.3.8 HPLC-ESI-MS Analysis Phenolic Acids**

A 2696 HPLC system (Waters Corp., Milford, MA) equipped with a model 996 photodiode array detector (PDA) (Waters) and model 717 plus autosampler (Waters) coupled with a quadrupole time-of-flight (Q-TOF) mass spectrometer (Micro Mass Waters, Milford, MA) was employed for HPLC-MS analysis with negative electrospray ionization. A 150 mm  $\times$  4.6 mm, 5  $\mu\text{m}$  RP 18 analytical column was used for separation (Gemini, Phenomenex, Torrance, CA). During the LC/MS analysis, 10  $\mu\text{l}$  of sample was loaded and injected by an autosampler, and eluted through the column with gradient mobile phase, then introduced to the Q-TOF. Mobile phase A for phenolic acids consisted of water containing 0.1% acetic acid and mobile phase B consisted of methanol containing 0.1% acetic acid. The 75 min-linear gradient for phenolic acids quantification was programmed as follows: 0-7 min, 15-20% B; 7-8 min, 20-15% B; 8-21 min, 15-24% B; 21-34 min, 24-13% B; 36-37 min, 13-20% B; 37-46 min, 20-42% B; 46-63 min 42-100% B; 63-75 mins, 100-15%B. Flow rates were set at 0.5 ml/min. The full mass spectra were recorded in negative mode by using the capillary voltage of 1.2 kV and cone voltage of 40 V. The flow rate of desolvation gas ( $\text{N}_2$ ) and cone gas were 900 L/h and 50 L/h, respectively. The desolvation temperature and the source temperature were set at 350°C and 150°C, respectively.

The MS/MS spectra were acquired by using collision energy of 30 V. The absorbance at 320 nm was used for quantification of phenolic acids. Vanillic acid was quantified at 291 nm as it showed maximum absorption at that wavelength. Ferulic, sinapic, caffeic, vanillic and p-coumaric acid were used as external standards for quantification of phenolic acids.

### **3.4 Results and Discussion**

#### **3.4.1 Sprouting**

Whole barley grains were sprouted for 19 hours in an automatic sprouter used at room temperature of 18.3 - 29.4°C. Sprouting was inhibited at the emergence of a white tip of the radical. During sprouting, grain swelling due uptake of water was observed. However, the four barley genotypes did not sprout at the same rate (**Figure 3.1**). CI2230 sprouted at a faster rate than Peru 35, CI1248 and EX127. Based on visual observation the rate of sprouting varied in the order CI2230 > Peru 35 > CI1248 > EX127. Barley grains from the same genotype did not germinate spontaneously. Water uptake is the first process that occurs during germination and is strongly influenced by the permeability of the seed coat (Lorenz 1980). The observations on sprouting suggest that the rate of water uptake among the genotypes and among grains within the same genotype varied. Temperature of germination has been reported to influence the rate of germination. The optimum temperature range in which sprouting occurs in barley ranges between 19 and 27 °C (Edward 1932). Genetic differences, age of seed, the source of seed and varietal differences will have an impact on the germination temperature as well. Generally cereals are known to show the “after-ripening” phenomenon. This phenomenon is characterized by slow germination of seeds at harvest but increases during storage (Lorenz 1980).

A



B



CI2230

EX127

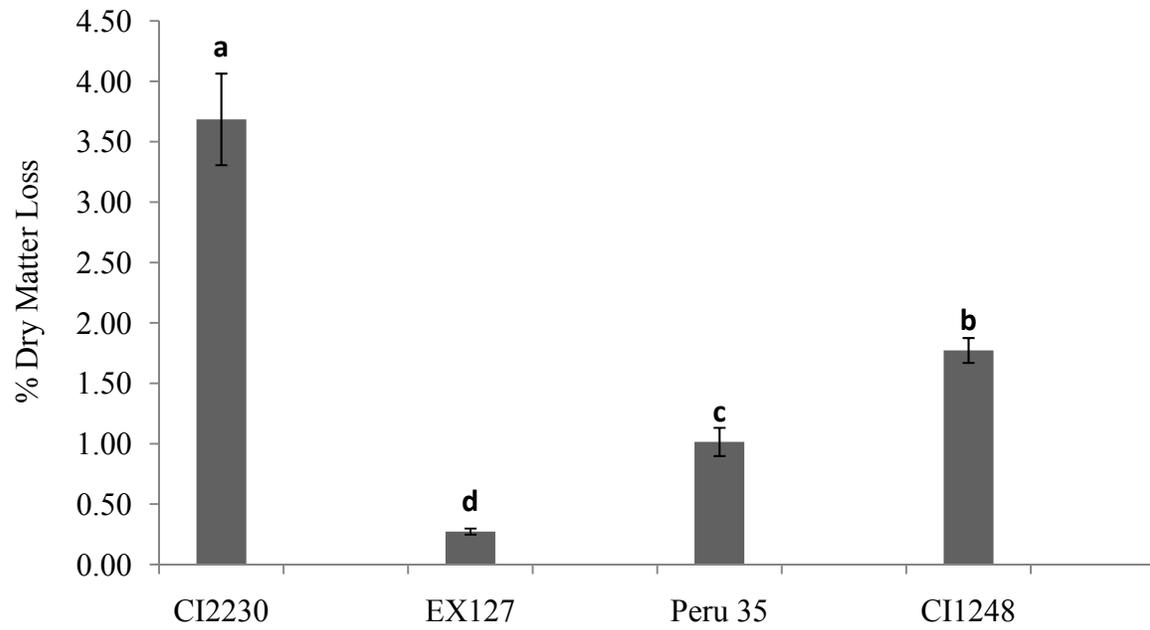
Peru 35

CI1248

**Figure 3.1: Pictures of (A) whole hull-less barley grains and (B) sprouted barley grains after 19 hours of sprouting in an automated sprouter.**

### ***3.4.2 Dry Matter losses during Sprouting***

Dry matter losses were observed in the barleys after 19 hours of sprouting (Figure 3.2). Overall, the barley grains lost an average 1.7% in dry matter. The highest loss in dry matter was observed in CI2230 (3.7%) while the lowest loss in dry matter was exhibited by EX127 (0.27%). Peru 35 and CI1248 had a loss in matter of 1.02% and 1.77% respectively. The loss in dry matter was significantly different among the partially sprouted barley genotypes ( $P < 0.05$ ). Chung et al (1989) observed a dry matter loss in sprouted barley. The loss was more dramatic in sprouted barley than sprouted canola seeds. Carbohydrate changes in the grain are due to increase in amylolytic activity (Mwikya et al 2001) and these changes have an impact on dry matter. During germination there is energy expenditure through catabolism of starch to sugars and lipids to free fatty acids and this contributes to dry matter losses. Respiration also accounts for part of the losses in dry matter through the escape of carbon dioxide and water from the grain. Sprouting is also associated with the increase in crude fibre and diglyceride in barley (Chung et al 1989). As reviewed by Chavan et al (1989), modifications in grain composition and nutritional content depends on cereal type, grain quality and sprouting conditions such as duration of sprouting. Bhise et al (1988) found a 19.5% loss in dry matter in sprouted sorghum after 92 hours. While Mwikya et al (2001) reported a loss of 15.9% loss in dry matter in sprouted kidney beans after 92 hours.



**Figure 3.2: Loss in dry matter in barley genotypes during partial sprouting. Measurements were performed in duplicate.**

### 3.4.3 Antioxidant Capacities of the Barley Genotypes

#### 3.4.3.1 Total Phenolic Content of Partially Sprouted Barley Genotypes

*Methanolic Extract* The reducing capacity of the barleys was determined using the total phenols assay using the Folin-reagent (**Table 3.1**). The TPC was expressed as ferulic acid equivalents (FAE). Ferulic acid, the major phenolic acid reported in barley was used as a standard (Nordkvist 1984). TPC of control barleys ranged from 2314 (CI1248) to 2844(CI2230)  $\mu\text{g/g}$ . The partially sprouted barleys had TPC ranging from 1784 (CI1248) to 2178 (CI2230)  $\mu\text{g/g}$  (**Table 3.1**). There were no significant differences between EX127 (2338  $\mu\text{g/g}$ ) and CI248 (2314  $\mu\text{g/g}$ ) raw barleys ( $P < 0.05$ ). Among the partially sprouted barleys, there were no significant differences between EX127 (1870  $\mu\text{g/g}$ ) and Peru 35 (2185  $\mu\text{g/g}$ ) ( $p < 0.05$ ). In the methanolic extract, CI2230, a Nepalese genotype displayed highest TPC for both the sprouted and raw barleys. While CI2230 and EX127 showed no significant difference between the sprouted and raw genotype, Peru 35 and CI1248 showed the reverse. Overall, raw barleys exhibited higher TPC than sprouted.

*Acetone Extract* Phenolic contents of the barley genotypes tested are presented in **Table 3.2**, expressed as micromoles of ferulic acid equivalents (FAE). TPC of the control samples ranged from 2856 (CI1248) to 4485(EX127)  $\mu\text{g/g}$  while that of the partially sprouted barley genotypes ranged from 2789 (EX127) to 4164 (CI2230)  $\mu\text{g/g}$  (**Table 3.2**). TPC of partially sprouted barleys was significantly higher than the representative raw barleys in the acetone extracts for the Nepalese and Israeli genotypes. Among the partially sprouted barleys, TPC significantly differed. While no significant differences were observed between Peru 35 and CI1248 in the raw barleys, TPC between CI2230 and EX 127 differed significantly ( $p < 0.05$ ). No significant

differences in TPC were found among the partially sprouted barleys and their respective controls except for EX127 which showed significantly higher TPC for control than the sprouted. The low TPC in the partially sprouted EX127 can be attributed to the degree of sprouting observed in the genotype (**Figure 3.1**) which can further be related to the loss in dry matter. The crude acetone extracts showed higher TPC than methanolic extracts. The difference in the TPC between the different crude extracts can be attributed to the selectivity and extraction capacity of solvents for specific barley phenolic compounds (Zhao et al 2006).

**Table 3.1 Antioxidant Activity of the Acetone Extracts of the Partially Sprouted Barley Genotypes and their Controls**

Barley Genotype	TPC ( $\mu\text{g FAE/g}$ )		DPPH ( $\mu\text{mol TE /100g}$ )		% Radical Scavenging Activity	
	<u>Sprouted Grain</u>	<u>Raw Grain</u>	<u>Sprouted Grain</u>	<u>Raw Grain</u>	<u>Sprouted Grain</u>	<u>Raw Grain</u>
CI2230	4164 $\pm$ 29.20a	3830 $\pm$ 3.55b	921 $\pm$ 22.25a	1062.37 $\pm$ 19.19a	27.24	31.35
EX127	2546 $\pm$ 82.92c	4485 $\pm$ 4.72a	881 $\pm$ 15.75a	928.65 $\pm$ 7.47a	26.24	27.56
Peru 35	2789 $\pm$ 56.66c	2992 $\pm$ 8.55c	886 $\pm$ 27.76a	1043.52 $\pm$ 59.41a	26.29	30.43
CI1248	3277 $\pm$ 155.08b	2856 $\pm$ 5.82c	951 $\pm$ 42.04a	1090.54 $\pm$ 98.44a	28.33	32.26
LSD	259.67	130.53	79.52	162.15	-	-

Mean values of samples having similar letters are not significantly different ( $P < 0.05$ );

TE, Trolox Equivalent

TPC, Total Phenolic Content

FAE, Ferulic Acid Equivalent

LSD denotes fisher's least significant difference.

### 3.4.3.2 DPPH Radical Scavenging Activities of the Partially Sprouted Barley Genotypes

*Methanolic Extract* Free radical scavenging activities of the sprouts and their controls are shown in **Table 3.1**. The reducing capacity of the crude extracts against the DPPH radical was measured using the DPPH assay. DPPH radical scavenging activity of the barleys was expressed as micromoles trolox equivalents per gram ( $\mu\text{mol TE/g}$ ) of whole barley and as percentage discoloration. The average DPPH radical scavenging activity varied from 527 (CI1248) to 786 (Peru 35) and from 598 (CI1248) to 645 (CI2230) for the control and partially sprouted barleys respectively. No significant differences were observed between the sprouted EX127 and CI1248 and between CI2230 and Peru 35. Significant differences were found between control and sprouted barleys for Peru 35, CI1248 and EX127. However, CI2230 displayed no significant differences in radical scavenging activity between control and partially sprouted barley. Sprouted Israeli genotype (CI1248) exhibited higher scavenging activity than control while the control barleys for EX127 and Peru 35 had higher activity than sprouted. The methanolic extract showed lower radical scavenging activity than acetone extract. The acetone extract exhibited the highest DPPH scavenging activity and TPC. The relatively low DPPH radical scavenging capacity suggests that the DPPH radical will only react with the more reactive phenolic compounds in the extracts since the DPPH radical is very stable (Stratil et al 2007).

*Acetone Extract* The hydrogen donating capacity of the phenolic compounds in the crude acetone extract was monitored spectrophotometrically at 515 nm after 30 mins of mixing. DPPH• scavenging activity at 30 mins ranged from 881(EX127) to 951 (CI1248) and from 929 (EX127) to 1091 (CI1248)  $\mu\text{mol TE/g}$  for the sprouted and control barleys respectively (Table 3.2). No significant differences were found among the control barleys in their radical scavenging activity. Similarly, the sprouted barleys did not differ significantly from each other. Peru 35, CI1248 and EX127 had no significant differences between the sprouted and control barleys. However, CI2230 was the only genotype that showed a significant difference between the sprouted and control barley. The control barleys were found to exhibit higher DPPH radical scavenging activity than the partially barley sprouts.

The raw grain generally exhibited significantly higher total phenolic content and DPPH radical scavenging activity than the partially sprouted genotypes, however, there were some exceptions. This observation can be attributed to the degree of sprouting (

**Figure 1.1).** The grains were partially sprouted and studies have shown that grains or legumes that are sprouted for longer times show a higher degree of modification (Khokhar et al 1986; Chavan et al 1989; Mwikya et al 2001). In this study, the grains were partially sprouted so as to maintain the intact grain structure rather than the traditional elongated sprouts sold commercially. Furthermore, the organic solvents employed for the crude methanolic and acetone extraction target mainly the free phenolic compounds. Yu et al (2001) reported on the extraction of most free phenolic acids in barley with hot water. Water used in sprouting process could have

resulted in the leaching of free phenolic compounds hence the low values reported for the partially sprouted genotypes (Yang et al 2001; Zhang et al 2004).

**Table 3.2: Antioxidant Activity of the Methanolic Extracts of the Partially Sprouted Barley Genotypes and their Controls**

Barley Genotype	TPC ( $\mu\text{g FAE /g}$ )		DPPH ( $\mu\text{mol TE /100g}$ )		% Radical Scavenging Activity	
	<u>Sprouted Grain</u>	<u>Raw Grain</u>	<u>Sprouted Grain</u>	<u>Raw Grain</u>	<u>Sprouted Grain</u>	<u>Raw Grain</u>
CI2230	2178 $\pm$ 205.93a	2844 $\pm$ 1.52a	645 $\pm$ 16.84a	677 $\pm$ 4.46b	38.53	40.25
EX127	1870 $\pm$ 121.41ab	2338 $\pm$ 68.63c	598 $\pm$ 3.32b	649 $\pm$ 5.62c	35.7	38.61
Peru 35	2185 $\pm$ 37.63a	2499 $\pm$ 57.57b	645 $\pm$ 3.21a	786 $\pm$ 1.15a	38.54	46.20
CI1248	1784 $\pm$ 94.21b	2314 $\pm$ 28.54c	600 $\pm$ 11.794b	527 $\pm$ 12.47d	35.93	31.02
LSD	360.51	130.53	29.37	20.04	-	-

Mean values of samples having similar letters are not significantly different ( $P < 0.05$ );

TE, Trolox Equivalent

TPC, Total Phenolic Content

FAE, Ferulic Acid Equivalent

LSD denotes fisher's least significant difference.

#### ***3.4.4 Identification and Quantification of Phenolic Acids in Partially Sprouted and Raw Barley Genotypes using HPLC analysis***

*Free Table 3.3* lists the quantified free phenolic acid in partially sprouted and control whole barley genotypes. Phenolic acid composition of the partially sprouted barleys and their respective controls was determined using an HPLC method. The phenolic acid content was expressed as micrograms per gram of whole barley. Ferulic acid was the major free phenolic acid that was quantified in the sprouted genotypes and their controls. Contents of free ferulic acid ranged from 1.59-3.69  $\mu\text{g/g}$  in both sprouted and unsprouted barleys. Among the raw barleys, the Nepalese genotype had the highest content of ferulic acid while no significant differences were observed among EX127, Peru 35 and CI1248. Although the contents of ferulic acids were low in sprouted barleys, the Nepalese genotype also displayed high contents. Free phenolic acids make the lowest contribution to the total phenolic acid content in cereals (Adom et al 2002). The low free phenolic acid contents reported in the partially sprouted genotypes can be attributed to the leaching of some of the phenolic acid during sprouting (Zhang et al 2004). Madhujith et al (2006) reported ferulic (0.86-3.57  $\mu\text{g/g}$ ) and vanillic (0.23-5.86  $\mu\text{g/g}$ ) as the major free phenolic acids in two barley cultivars. Yang et al (2001) showed a decrease in phenolic acid content and this was attributed to the leaching out of water-soluble free phenolic acids in wheat grain during the steeping process.

**Table 3.3: Content of Free Acids in Partially Sprouted and Raw Barley Genotypes**

Barley Genotype	Free Phenolic Acids	
	Sprouted	Raw
CI2230	2.51±0.16a	3.69±0.21a
EX127	1.77±0.13bc	2.40±0.01b
Peru 35	1.94±0.00b	2.53±0.13b
CI1248	1.59±0.09c	2.21±0.14b
LSD	0.31	0.98

Free phenolic acid content is expressed in micrograms per gram of whole barley.

Each value is the mean± standard deviation of duplicate determination.

Mean values of samples having similar letters are not significantly different ( $P < 0.05$ ).

LSD denotes Fisher's least significant difference.

*Soluble conjugated* Alkaline hydrolysis is the method employed to release bound or esterified phenolic acids at room temperature. Soluble conjugated phenolic acids made up a greater portion of the total phenolic acid content in the barley genotypes. Error! Reference source not found. lists the contents of esterified phenolic acid that were liberated upon alkaline hydrolysis at room temperature under nitrogen. The soluble conjugated acids identified at 320

nm in the raw and sprouted barleys included ferulic, *p*-coumaric, caffeic and sinapic. Vanillic acid (VA) was the only hydroxybenzoic acid derivative identified and was quantified at 291 nm.

The monomeric ferulic acid (FA) was the most abundant phenolic acid in the soluble conjugated fraction. Total FA content in partially sprouted and raw barley was 213.8 and 196.3 µg/g respectively. Sprouting increased the content of soluble conjugated ferulic acid in the barley genotypes. However, differences in FA content between the treatments were minor despite the significant difference between the dry matter contents. Ferulic acid in the raw and sprouted barleys ranged from 38.8 to 63.6 µg/g. Among the raw grains there were no significant differences between EX127 (38.8 µg/g) and C1248 (40.3 µg/g). CI2230 displayed highest contents of ferulic acid among the raw and sprouted grains. The high contents of ferulic acid in the soluble conjugated phenolic acid fraction of partially sprouted barley can be attributed to the release of the ferulic acid that is esterified with arabinose in plant cells through enzyme activity initiated by sprouting (Mwikya 2001).

*p*-Coumaric acid (*p*-CA) was the second most abundant soluble conjugated phenolic acid quantified in raw and sprouted grains. Total *p*-CA contents in sprouted and raw barley were 30 and 33.3 µg/g respectively. Sprouting did alter the *p*-CA contents. The raw barleys (controls) had high *p*-CA contents for all genotypes. The highest *p*-CA content was exhibited by EX127 for both sprouted (8.88 µg/g) and raw grain (10.4 µg/g) among the sprouted genotypes, Peru 35, EX127 and Peru 35 did not differ significantly. Hulled varieties of barley have been reported to have high *p*-CA content compared to hull-less (Hernanz et al 2001, Holtekjolen et al 2006). The genotypes used for this study were all hull-less. Dvorakova et al (2008) reported quantities of *p*-

CA ranging between 1.2-1.7  $\mu\text{g/g}$  in 10 spring barley varieties. The contents of soluble conjugated *p*-CA reported by Dvorakova et al (2008) are low compared to those observed in this study.

Partially sprouting the barley genotypes significantly increased the contents of CA. Total CA contents in sprouted and raw barleys was 80.3 and 17.9  $\mu\text{g/g}$  respectively. The Nepalese sprouted genotype (35.6  $\mu\text{g/g}$ ) exhibited the highest CA content. Sprouted EX127 (11.4  $\mu\text{g/g}$ ), Peru 35 (17.2  $\mu\text{g/g}$ ) and CI1248 (16.2  $\mu\text{g/g}$ ) showed no significant differences in their CA contents ( $P < 0.05$ ). Among the control genotypes no significant differences were observed. There was a significant increase the CA content as a result of the partial sprouting. CA has been found to possess antitumor activity against colon cancer hence consumption of sprouted barley can be of health benefit (Oltholf et al 2001).

VA was the least abundant soluble conjugated phenolic acid in the sprouted grains. It was not quantified in all the raw grains except in EX127 (3.91  $\mu\text{g/g}$ ) as the levels were too low for quantification. Total VA contents for the sprouted and raw grain were 14.8 and 3.91  $\mu\text{g/g}$  respectively. Quantities of VA ranged from 3.12 (Peru 35) - 4.27 (CI2230)  $\mu\text{g/g}$  in sprouted genotypes. No significant differences were exhibited between the mixed (EX127) and Peruvian genotypes and between the Israeli (CI1248) and Nepalese (CI2230) sprouted genotypes. VA has been identified and quantified in two barley cultivars (Madhujith et al 2006). Dvorakova et al (2008) also reported quantities of VA ranging between 2.1-3.6  $\mu\text{g/g}$  in 10 spring barley varieties.

Sinapic acid (SA) was not identified in free phenolic acids fraction but was identified in the soluble alkaline esters after hydrolysis. Total SA contents for the sprouted and raw grain

were 235 and 18  $\mu\text{g/g}$  respectively. Sprouting was found to significantly increase the SA content among all genotypes. CI2230 (93  $\mu\text{g/g}$ ) exhibited the highest content of SA. However, SA was not quantified in the raw barley genotypes, Peru 35 and EX127 due to the low levels that made quantification challenging. Content of sinapic acid varied from 33 (CI1248) to 93 (2230)  $\mu\text{g/g}$  in the partially sprouted barley genotypes. Sprouted barleys differed significantly in their SA contents ( $P < 0.05$ ). Dvorakova et al (2008) reported quantities of soluble conjugated SA ranging between 5.1-7.6  $\mu\text{g/g}$  in 10 spring barley varieties. Madhujith et al (2006) also reported soluble conjugated SA contents ranging between 0.48-70  $\mu\text{g/g}$  in 2 barley cultivars. The contents reported in literature are for raw barley grains. The contents obtained from sprouting show a means of improving the phenolic content in our diets and thereby increasing the subsequent health benefits.

Table 3.4: Content Soluble Conjugated Phenolic Acids in Partially Sprouted and Raw Barley Genotypes

Barley Genotype	Soluble Conjugated Phenolic Acids									
	Ferulic		<i>p</i> -Coumaric		Caffeic		Sinapic		Vanillic	
	Sprouted	Raw	Sprouted	Raw	Sprouted	Raw	Sprouted	Raw	Sprouted	Raw
CI2230	63.6±5.61a	63.1±1.52a	5.79±0.23b	6.84±0.01c	34.2±5.78a	4.45±0.62a	93.3±24.39a	10.3±1.02	4.25±0.10a	nd
EX127	49.0±0.23b	38.8±0.47c	8.88±1.09a	10.4±1.24a	9.66±3.10b	4.18±0.77a	35.1±40.41b	nd	3.23±0.18b	3.91±0.70
Peru 35	46.9±1.49b	40.3±2.10c	7.11±0.78ab	7.67±0.23b	16.2±3.35b	4.27±0.22a	44.1±1.37c	nd	3.05±0.26b	nd
CI1248	54.3±4.31ab	54.1±1.75b	8.22±1.03ab	8.37±0.06b	16.2±1.48b	4.06±0.58a	33.4±0.44d	7.28±0.07	4.13±0.22a	nd
Total	213.8	196.3	30.0	33.3	80.3	17.9	235	17.8	14.8	3.91
LSD	10.0	4.16	2.20	0.98	10.66	1.39	9.46	2.6	0.66	

Soluble conjugated phenolic acid content is expressed in micrograms per gram of whole barley

nd, not detected

Each value is the mean± standard deviation of duplicate determination

Mean values of samples having similar letters are not significantly different ( $P < 0.05$ ).

LSD denotes Fisher's least significant difference.

*Insoluble Bound* FA was the most abundant insoluble bound phenolic acid in both the sprouted Peruvian genotype (1547 µg/g) and raw Nepalese (1740 µg/g) genotype. Total FA contents of sprouted and raw barleys were 7490 and 6835 µg/g respectively. Barley sprouts exhibited more FA in terms of total ferulic acid content (**Table 3.5**). The raw barley genotypes used in this study showed contents ranging from 933(EX127)-2406(Peru 35) µg/g. Raw barleys (controls) for CI2230 and CI1248 had relatively higher FA contents than sprouted barley. Peru 35 exhibited the highest FA contents for sprouted and control grains. Among the sprouted genotypes, CI2230 and CI1248 did not differ significantly. The insoluble bound FA extracts for barley (sprouted and raw) exhibited high FA in comparison to wheat and rye after alkaline hydrolysis. FA contents ranging from 784 to 1980 µg/g in durum wheat have been reported (Lempereur et al 1998). While rye has been reported to contain 859 to 1174 µg/g (Andreasen et al 2000).

*p*-CA, the second most abundant phenolic acid showed total contents of 298 and 295µg/g for sprouted and control barleys respectively (**Table 3.5**). Peru 35 also exhibited the highest concentrations of *p*-CA for both sprouted and raw grains. Contents of *p*-CA varied significantly among the sprouts and raw grains. In partially sprouted and raw barleys, *p*-CA ranged from 28 to 112 and 40-133 µg/g respectively. The general trend observed showed that the raw barleys had significantly higher insoluble bound *p*-CA contents except for EX127 that showed otherwise. Dvorakova et al (2008) reported contents of *p*-CA ranging from 18 to 52 µg/g in 10 barley spring varieties. Quinde-Axtell et al (2006) reported quantities of *p*-CA that ranged from 4 to 68 µg/g in 11 barley genotypes depending on presence or absence of hulls. Differences in the *p*-CA contents can be attributed to variations in extraction methods. Hydrolysis procedures have an effect on the phenolic acids detected (Ross et al 2009).

There were no significant differences between the caffeic acid (CA) contents of both sprouted and control barleys ( $P < 0.05$ ) among genotypes. The total concentrations of bound CA for barley sprouts and raw grain were 82 and 112  $\mu\text{g/g}$  respectively. Contents of CA in raw barley were significantly higher ( $P < 0.05$ ) than that of barley sprouts. Among the barley sprouts, the highest and lowest contents of CA were observed in Peru 35 (23  $\mu\text{g/g}$ ) and EX127 (18  $\mu\text{g/g}$ ) respectively. The low contents of CA exhibited by EX127 can be attributed to the low dry matter loss indicating minimal modification occurred during sprouting. While the highest and lowest contents for raw grains were observed in CI2230 and CI1248 respectively.

The changes observed in the contents of free, soluble conjugated and insoluble bound phenolic acids upon sprouting can be explained by either the possible action of induced esterases on phenolic acid-polysaccharide or phenolic acid-protein complexes thereby releasing the phenolic acids reported. Furthermore these changes can be attributed to the de novo synthesis of phenolic acids, followed by their storage in seeds instead of migrating to shoots (Subba Rao et al 2002).

**Table 3.5: Content of Insoluble Bound Phenolic Acids in Partially Sprouted and Raw Barley Genotypes**

Barley Genotype	Insoluble Bound Phenolic Acids					
	<u>Ferulic</u>		<u>p-Coumaric</u>		<u>Caffeic</u>	
	Sprouted	Raw	Sprouted	Raw	Sprouted	Raw
CI2230	1363±3.23b	1740±186.53b	28.2±0.08c	55.2±7.14bc	21.7±0.20a	33.7±6.05a
EX127	2325±77.1a	933±183c	112.3±4.87a	39.7±13.4c	18.1±2.89a	25.5±5.56a
Peru 35	2279±66.7a	2406±287.67a	109.6±5.77a	133.6±66.28a	22.8±3.04a	31.2±15.63a
CI1248	1523±224.02b	1757±104.22b	47.7±7.27b	66.3±1.68b	19.4±1.54a	21.1±14.88a
Total	7490	6835.1	297.8	294.7	82.0	111.6
LSD	341.71	559.19	14.5	15.88	6.2	18.12

Insoluble bound phenolic acid content is expressed in micrograms per gram of whole barley

Each value is the mean± standard deviation of duplicate determination

Mean values of samples having similar letters are not significantly different (P<0.05).

LSD denotes Fisher's least significant difference

### **3.5 Conclusion**

Modification of phenolic compounds in grains as a result of sprouting may be beneficial. Sprouting was observed to increase the content of mostly soluble conjugated phenolic acids in barley which include ferulic, p-coumaric, caffeic, vanillic and sinapic acid and this was accompanied by losses in dry matter. Minor differences in bound phenolic acids due to sprouting were observed between sprouted and raw barleys. However, levels of free phenolic acids decreased with sprouting. The Peruvian genotype displayed high contents of bound and soluble conjugated phenolic acids. Barley sprouts present a novel food ingredient with increased soluble conjugated phenolic acids.

## ***Chapter 4. References***

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