Identification and Antioxidant Properties of Phenolic Compounds during Production of Bread from Purple Wheat Grains and Investigation of Bread Extracts after Simulated Gastrointestinal Digestion

By

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ABSTRACT

Content of free- (FPC) and bound- phenolics (BPC) significantly ($p<0.05$) increased during mixing, fermenting and baking. Bread crust and crumb contained the highest FPC and BPC, respectively. Antioxidant activities (AOA) followed the trends of their respective phenolic contents. HPLC analysis demonstrated that different phenolic acids showed various responses to the bread-making process. Total anthocyanin content (TAC) was significantly ($p<0.05$) reduced through mixing and baking, but fermentation elevated the levels. Anthocyanin extract of purple wheat exerted higher AOA than those of common wheat. Digested purple wheat extracts after *in-vitro* digestion demonstrated significantly ($p<0.05$) higher AOA than common wheat. During *in-vitro* testing, extracts exhibited concentration-dependent effects, while the use of different cell lines exhibited varying levels of cellular antioxidant and pro-oxidant properties. Purple wheat demonstrated higher cytoprotectivity and cellular AOA than those of common wheat. Our findings suggest that purple wheat has the potential to act as functional food in bakery products.

Key words: purple wheat, bread-making, phenolic compounds, anthocyanins, antioxidant activity, *in-vitro* digestion, cell culture

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LIST OF ABBREVIATIONS

AACC American Association of Cereal Chemists International
AAPH 2,2'-azobis (2-amidinopropane) dihydrochloride
ABTS 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
AOA Antioxidant activity
AOAC Association of Official Analytical Chemists International
ATCC American Type Culture Collection
BPC Bound phenolic content
C3GE Cyanidin-3-glucoside equivalents
CAA Cellular antioxidant activity
CSP Canadian short process method
DCFH-DA Dichlorofluorescin diacetate
DMEM Dulbecco’s Modified Eagle Medium
DPPH 2,2-Diphenyl-1-picrylhydrazyl
ECCC European Collection of Cell Cultures
FAB Farinograph absorption for baking
FAE Ferulic acid equivalents
FBS Fetal bovine serum
FPC Free phenolic compounds
FRAP Ferric reducing/antioxidant power
GAE Gallic acid equivalents
GI Gastrointestinal
GRL Grain Research Lab
HPLC Reversed phase high performance liquid chromatography
hTF Human transferrin
MRPs Maillard Reaction Products
MS Mass spectrometry
MS/MS Tandem mass spectrometry
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide
ORAC Oxygen radical absorbance capacity
PBS Phosphate buffered saline
PDA Photodiode array detection
Q-TOF-MS Quadrupole time-of-flight mass spectrometer
ROS Reactive oxygen species
SD Standard deviation
SET Single electron transfer
TAA Total antioxidant activity
TAC Total anthocyanin content
TE Trolox equivalents
TEAC Trolox equivalent antioxidant capacity
TPC Total phenolic content
TRAP Total radical absorption potential

GENERAL INTRODUCTION

In recent years, research activities have been directed towards the investigation of anti-inflammatory, anti-hepatotoxic and anti-carcinogenic effects of antioxidants (Kessler et al., 2003). Polyphenolic compounds are of particular interest because of their widespread consumption in human diet. One of the major functional properties of phenolic compounds is to scavenge free radicals in organisms, therefore having potential to perform disease-preventing or health-promoting effects. Epidemiological studies have shown an inverse relationship between the consumption of whole grain based products and risks of chronic disease resulting in documentation of their potential health effects in recent reviews (Okarter and Liu, 2010; Munter et al., 2007). These chronic diseases include cardiovascular diseases, type II diabetes, obesity and some cancers.

Phenolic acids are a major category of phenolic compounds present in cereal grains. They have been well investigated. Anthocyanins are water soluble pigments mainly located in the bran and aleurone layers of a kernel, responsible for the natural color of cereal grain, such as purple, red and blue. They are powerful antioxidants, contributing significantly to the antioxidant activity of pigmented cereal grains. Increased interest in investigating anthocyanins is attributed by their potential as functional foods and nutraceutical ingredients. Recently, consumption of varieties of cereal grains with colored pericarp is particularly famous and products made from pigmented cereal grains are largely valuable in the marketplace.

Baking is a complex process where physical and chemical changes occur simultaneously in flour dough. It may alter the levels of phenolic compounds, and also modify their

antioxidant activities, leading to changes in the potential health benefits of the final product. Moreover, a better understanding of the critical steps in bread production in relation to these bioactive compounds will help to limit phenolic degradation during manufacturing, thus improving the functional potentials of final products. To date, there is very limited literature on evaluation of the effects of bread-making on phenolic acids and anthocyanins. Therefore, the present study aimed to evaluate the evolution of phenolic acids and anthocyanins during the production of bread, with purple wheat varieties.

The beneficial effects of cereal phenolic compounds are also dependent on their bioavailability and metabolic fate in human gut. Most studies on phenolic properties mainly focused on isolated compounds from food substances using organic solvent extraction procedures that are not representative of the human gut conditions prevailing during digestion. *In-vitro* gastrointestinal (GI) tract models are widely used for investigating food components under simulated gastrointestinal conditions. These models simulate the digestion in human gut and are basically constructed upon starch digestion by amylases, lipid digestion by lipases and protein digestion by pepsin or trypsin. They have been regarded as simple, inexpensive, and reproducible systems for studying various food compounds. Cell cultures are also widely used techniques for examining the cellular functional properties of phenolic compounds. However, most studies utilized a pure food component or organic solvent-extracted compound. Their cellular antioxidant activities are considered to differ from the extracts after digestion using simulated GI tract model. Therefore, the present study employed *in-vitro* digestion model, aiming to examine the functional properties of purple wheat bread after gastrointestinal digestion using chemical and cell culture approaches.

CHAPTER I. Literature Review

1.1. Introduction

Cereals are staple foods for most of the world's population and provide energy, proteins, dietary fibre, minerals, and vitamins that are vital for human health (Liu, 2007). About 50% of the dietary fibre consumed in Western countries comes from cereals such as wheat, maize, barley, oat and rye (Vitaglione et al., 2008). Numerous studies suggest that consumption of whole–grain cereals is beneficial in the prevention of oxidative-stress related chronic diseases and metabolic disorders (Quideau et al., 2011; Okarter et al., 2010; Jacobs et al., 1998). The health potential is partly attributed to the antioxidant properties of phenolic compounds naturally present in whole grains (Adom et al., 2005; Zielinski & Kozlowska 2000). However, cereals are processed before they are consumed or used as a food ingredient. Such processing may alter the functional properties of phenolic compounds. This chapter constitutes a literature review of phenolic compounds and the effects of processing on the properties of antioxidant compounds in wheat grains.

Various analytical methods have been used in identifying the antioxidant properties of phenolic compounds in cereals. These methods include *in-vitro*, *ex-vivo* and *in-vivo* assays. *In-vitro* studies are usually conducted in test tube, which can simplify the reaction and focus on a small number of components (Vignais and Vignais, 2010). However, the information on antioxidant effectiveness in organisms is limited since chemical assays neither reflect cellular physiological conditions nor consider the bioavailability, uptake and metabolism of antioxidants in organisms (Mermelstein, 2008). In contrast, *in-vivo* animal models attempt to provide physiological conditions that closely resemble those of humans; however, they are expensive and time consuming. Therefore, cultured cells derived from biopsies have been

used in evaluating the antioxidant properties of phenolic compounds. They are relatively low cost compared to the *in-vivo* assays and may give more insightful information beyond *in-vitro* chemical assays. The ability of polyphenols to protect organisms from oxidative stress has been well documented in various *in-vitro* and cell culture models (Bueno et al., 2012; Movahed et al., 2012; Khanduja and Bhardwaj, 2003). However, some phenolic compounds have been reported to be pro-oxidant (Matsuo et al., 2005; Babich et al., 2009; Prochazkova et al., 2011). For example, tannic, ellagic, gallic acid and quecitin have been reported to possess both antioxidant and pro-oxidant properties (Labieniec and Gabryelak, 2007; Lee et al., 2003). Factors that lead to the contradictory properties of phenolic compounds require further investigation. The present chapter also discusses *in-vitro* assays and cell culture models, and explores factors that contribute to the difference in *in-vitro* and *ex-vivo* analysis.

1.2. Phenolic Compounds

1.2.1. *Classification of Phenolic Compounds*

Phenolic compounds are natural antioxidants commonly found in fruits, vegetables (Gercia-Salas et al., 2010) and cereal grains (Qiu et al., 2010; Liu et al., 2010; Bellido and Beta, 2009; Hosseinian et al., 2008). The classification of phenolic compounds is illustrated in **Figure 1.1**.

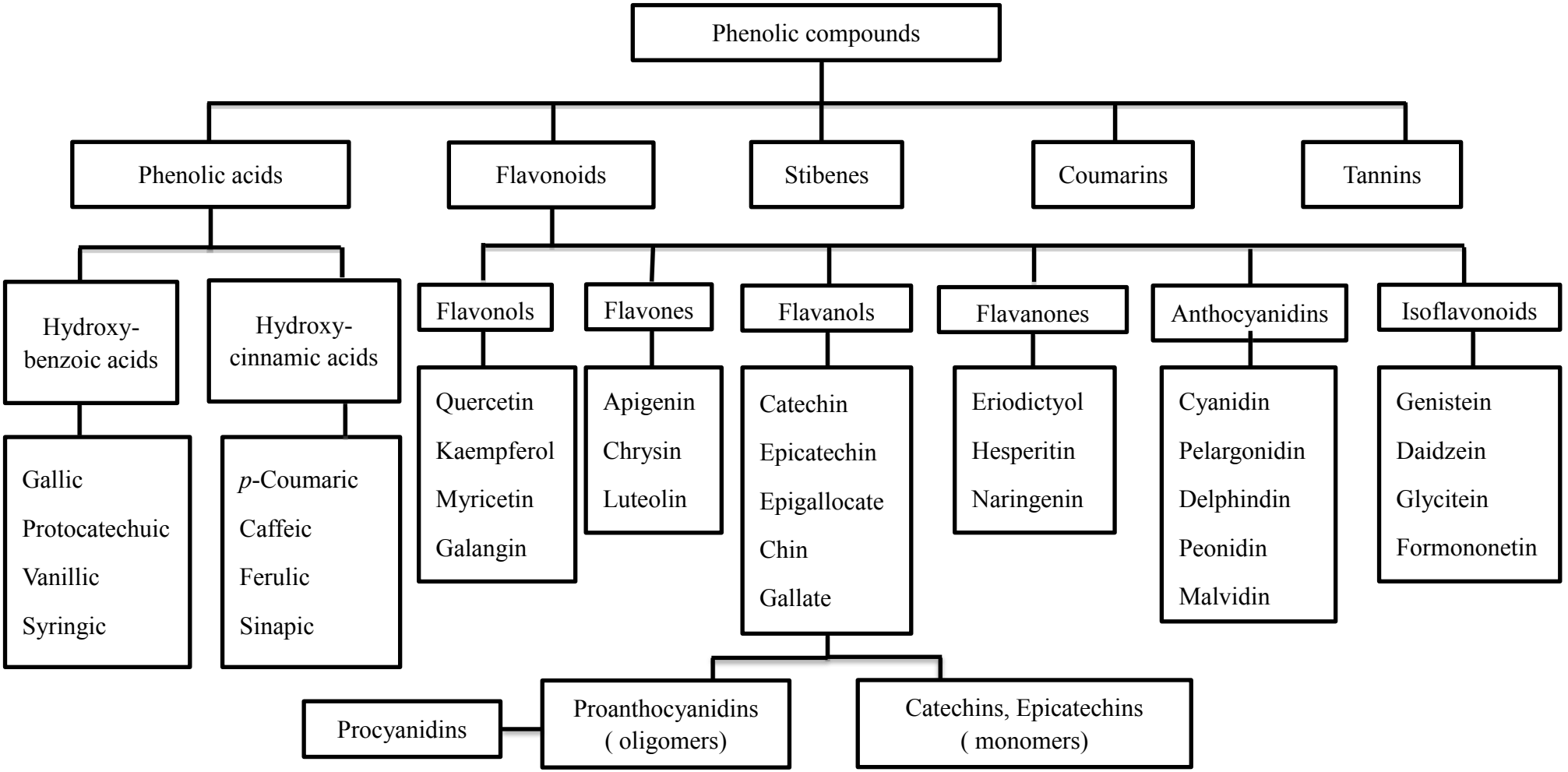


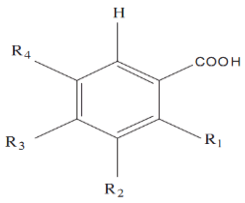
Figure 1.1 Classification of phenolic compounds (Liu, 2004).

Phenolic acids and flavonoids are the major phenolic compounds, and make up 60% and 30% of human total dietary polyphenols, respectively (Nichenametla et al., 2006). Plant polyphenols play important roles in food properties such as color, bitterness, astringency (Haslam, 2007), and a range of tactile or mouth-feel characteristics (Lesschaeve and Noble, 2005). For example, anthocyanins, a subclass of flavonoids are responsible for the color of various fruits, vegetables and pigmented cereals (Bueno et al., 2012). This color may vary depending on the pH of the cellular juice and the microelements with which they are combined (Lengyel et al., 2012).

1.2.2. Phenolic Acids

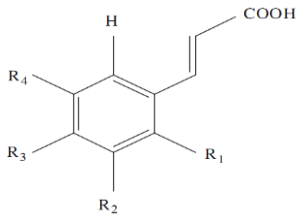
1.2.2.1. Classification and chemical structures of phenolic acids in wheat

Phenolic acids are classified into hydroxycinnamic and hydroxybenzoic acids (Garcia-Conesa et al., 1999). The former is composed of ferulic, sinapic, *p*-coumaric and caffeic acid while the later comprise gallic, protocatechuic, vanillic and syringic acid (**Figure 1.2)**.



Hydroxybenzoic acids

Name	R_1	R_2	R_3	R_4
Gallic acid (GA)	H	OH	OH	OH
Vanillic acid (VA)	H	OCH_3	OH	H
Protocatechuic acid (PRCA)	H	OH	OH	H
Syringic acid (SRA)	H	OCH_3	OH	OCH_3
Salicylic acid (SLA)	OH	H	H	H



Hydroxycinnamic acids

Name	R_1	R_2	R_3	R_4
Cinnamic acid (trans-CA)	H	H	H	H
p-coumaric acid (*p*-CA)	H	H	OH	H
Ferulic acid (FA)	H	OCH_3	OH	H
Caffeic acid (CA)	H	OH	OH	H
Sinapic acid (SA)	H	OCH_3	OH	OCH_3

Figure 1.2 Hydroxycinnamic and hydroxybenzoic acids (Irakli et al., 2012).

1.2.2.2. Distribution and forms of phenolic acids in wheat

Ferulic, *p*-coumaric, vanillic, caffeic, syringic, sinapic and protocatechuic acids are the common phenolic acids found in whole wheat grains (Vitaglione et al., 2008; Guo and Beta, 2013). Among these, ferulic acid is the most predominant (Beta et al., 2005) and is concentrated in bran (Vitaglione et al., 2008) and aleurone layer of wheat (Parker et al., 2005). Vanillic acid is the second abundant phenolic acid in wheat bran followed by syringic acid and *p*-coumaric acid (Kim et al., 2006).

Some of these phenolic acids are part of the structural components and protective systems (Bunzel et al., 2000; Renger and Steinhart, 2000). Depending on their existence, phenolic acids can be further categorized as: free phenolic acids, acids in soluble ester bonds and acids occurring in insoluble complexes (Hatcher and Kruger, 1997; Klepacka and Fornal, 2006). Zhang et al. (2012) investigated the free and bound type of phenolic acids in 37 Chinese winter wheat cultivars. They concluded that free forms made up of 2.5% of total phenolic acid content, among which syringic acid accounted for 44.7%, whereas bound phenolic acids constituted 97.5% (661 ug/g) with 70.7% being ferulic acid. Most bound phenolic acids are associated with mono- or poly-saccharides (Garcia-Salas et al. 2010; Lin and Harnly, 2007). For example, ferulic acid is bound to arabinoxylans through ester-bond (Garcia-Conesa et al., 1999; Rondini et al., 2004) or ether-linked to lignin or lignin-like polymers (Vaidyanathan and Bunzel, 2012). Parker and his group (2005) found that 95% of wall-bound esterified phenolics were ferulates and 39% of these were dehydrodiferulic acids. In the same study, the authors also indicated that the presence of dehydrodiferulic acids provided explanation for the protective nature of bran and the resistance to digestion of dietary fibre. The presence of linked ferulic acid is responsible for the formation of diferulates, which is a bridge structure

between cell wall polysaccharides (**Figure 1.3**).

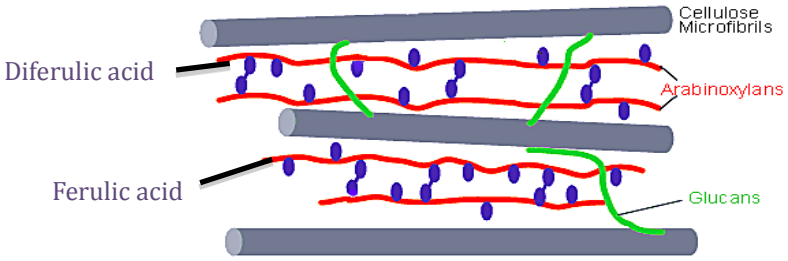


Figure 1.3 Bridge structure of diferulic acid between cell wall polysaccharides (Bunzel et al., 2001).

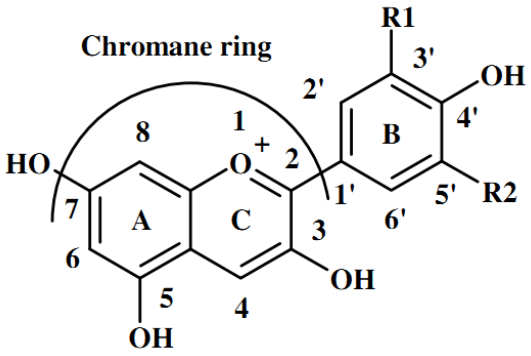
These cell wall polysaccharides prevent the phenolic acids from digestion in the small intestine. However, these acids are released in the large intestine during fermentation (Pandey and Rizvi, 2009). These non-fermentable phenolic compounds may remain in the human colon and help in quenching the free radicals, and therefore contribute towards prevention of some chronic diseases (Palafox-Carlos et al., 2011).

1.2.3. Anthocyanins

1.2.3.1. Profile and chemical structure of anthocyanins in colored wheat

Anthocyanins are pigments responsible for red, blue and purple colors of fruits, vegetables and cereal grains (Hosseinian and Beta, 2007). Unlike red and white wheat, which only contain very small amounts of anthocyanin compounds, larger amounts of anthocyanins are present in blue and purple wheat, being concentrated in the aleurone and pericarp layers, respectively (Abdel-Aal et al., 2006; Dykes and Rooney, 2007). Although these pigments exist in wheat kernels at low concentrations, it is assumed that this coloration may be used to distinguish the health benefits and market value of pigmented wheat versus common wheat products.

The concentration of anthocyanins reaches maximum level during grain development and then decreases to minor level after maturity (Knievel et al., 2009). The most common anthocyanidin found in plants is cyanidin, followed by delphinidin, peonidin, perlargonidin, petunidin and malvidin (Oomah and Mazza, 1999). The basic structures of anthocyanins and their colors as summarized by Hosseinian et al. (2008) are shown in **Figure 1.4**.



Aglycone	R1	R2	Color	λ_{max}(nm)
Cyanidin	OH	H	Red	535
Peonidin	OCH_3	H	Bluish-purple	532
Pelargonidin	H	H	Orange-red	520
Malvidin	OCH_3	OCH_3	Purple	542
Delphinidin	OH	OH	Purple	546
Petunidin	OCH_3	OH	Purple	543

Figure 1.4 Common anthocyanin structure and corresponding anthocyanidins (aglycones) (Hosseinian et al., 2008)**.**

Anthocyanins exist in plants in glycosylated forms, for example, linked with glucose, galactose, fructose, arabinose, rhamnose and xylose (Choia et al., 2007; Hosseinian and Beta, 2007). Research has demonstrated that the predominant anthocyanins in purple wheat mainly consist of cyanidin-3-glucoside, peonidin-3-glucoside, and cyanidin-3-galactosidedepending on the genotype (Dedio et al., 1972; Abdel-Aal and Hucl, 2003; Abdel-Aal et al., 2006; Knievel et al., 2009). Hosseinian et al. (2008) also reported the existence of other types of

anthocyanin compounds in lower concentrations. In general, the total anthocyanin content is affected by the genetics, light, temperature, and agronomic conditions (Majoul et al., 2003; Jing et al., 2007). Magnesium fertilization and early harvest have been reported to effectively increase the content of anthocyanins in purple wheat (Bustos et al., 2012).

Anthocyanins are relatively unstable and undergo reversible structural transformations at different pH conditions (Prior and Wu, 2006). When in solution, different forms of anthocyanins, namely flavylium cation, quinoidal base, hemiacetal base and chalcone, build equilibrium among themselves (Prior and Wu, 2006) as illustrated by Lee et al. (2005) (**Figure 1.5)**.

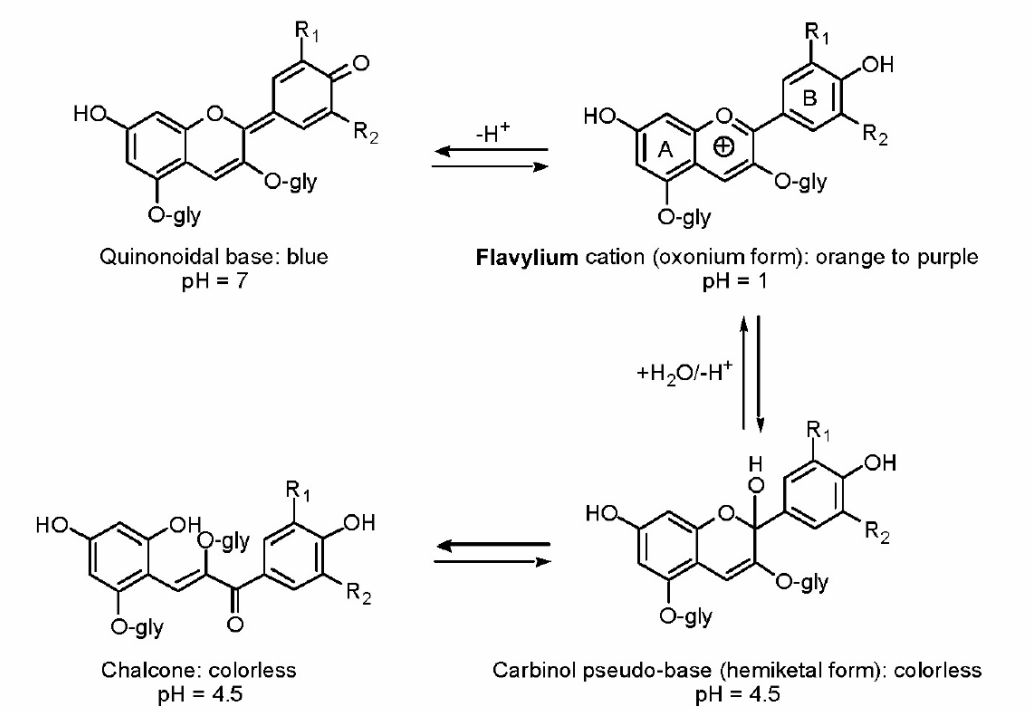


Figure 1.5 Predominant structures of anthocyanins at different pH (Lee et al., 2005)

1.2.3.2. Functional properties of anthocyanins

The increase in number of studies on anthocyanin functional properties in the past few decades suggests their potential benefits (Lila, 2004; He and Giusti, 2010). Anthocyanins

have potential to exert anti-obesity, anti-inflammatory and anti-cancer effects (Prior and Wu, 2006). Anthocyanins are also known to contribute to the beneficial roles on cardiovascular health, oxidative damage, detoxification enzymes and immune system (Manach et al, 2005). Pigmented wheat demonstrated higher antioxidant properties compared to white grained varieties. Li et al. (2005) reported that purple and blue wheat had higher radical scavenging capacity than white wheat. Hu et al. (2007) subsequently indicated that 69% of the overall free radical scavenging capacity of dark blue wheat grains was contributed by anthocyanins, whereas the extractable phenolic acids were only responsible for 19%. In addition, anthocyanins have shown their ability to suppress both hydrogen peroxide-induced oxidation and bacterial lipopolysaccharide-induced nitric oxide in cell culture models (Hu et al., 2007). Anthocyanins also exert protective effects through other antioxidant mechanisms, such as metal chelation and protein binding (Kong et al., 2003). A study done by Abdel-Aal et al. (2008) also indicated that the molecular structure of anthocyanins has important impact on their antioxidant properties. For example, cyanidin-containing anthocyanins showed higher scavenging capacities compared to delphinidin-based anthocyanins, which on the contrary possessed higher inhibitory capacity against copper-induced human LDL oxidation. Therefore, it may be inferred that pigmented wheat has the potential as a novel food ingredient to develop functional foods.

1.3. Effect of Bread-making Process on Phenolic Compounds in Wheat Flour

Food processing is often considered to have both positive and negative effects on phenolic compounds in whole grains and grain products. One such product is bread. It is

therefore assumed that bread-making may affect the wheat phytochemicals. Bread-making is a complex process during which the physical and chemical changes may occur in the dough and bread. Hansen et al. (2002) investigated the changes in ferulic acid in rye wholemeal during bread-making process. The total ferulic acid significantly decreased after mixing and maintained a similar level during proofing and baking while free ferulic acids increased throughout the process of mixing, proofing and baking. Results from a recent study using chemical assays showed a reduction in total phenolic content in bread compared to whole wheat flour (Angioloni and Collar, 2011). This finding is in agreement with several previous studies reported in the literature (Leenhardt et al., 2006; Alvarez-Jubete et al., 2010). The decrease in phenolic content could be explained by the presence of oxidative enzymes (oxygenase and peroxidase) that are activated when water is added to the flour (Angioloni and Collar, 2011). In addition, continuous incorporation of oxygen into the dough during processing facilitates the oxidation of phenolic compounds through combined reactions which are catalyzed by lipoxygenase (Eyoum et al., 2002). Moreover, baking may also lead to degradation of phenolic compounds (Angioloni and Collar, 2011), since some are reported to be thermally labile (Han and Koh, 2011). For example, a 2012 study identified the thermal stability of natural antioxidants, and concluded that the stabilities of selected antioxidants were in decreasing order of alpha-tocopherol > caffeic acid > ferulic acid > gallic acid (Santos et al., 2012). The study also observed that the decomposition temperature for gallic acid was lower than 110 ^{o}C (Santos et al., 2012). On the contrary, Gelinas and McKinnon (2006) reported that baking slightly increased the concentration of phenolic compounds and the crust of white bread contained slightly more phenolic compounds than the crumb. Slavin,

Jacobs and Marquart (2000) also indicated that the formation of Maillard reaction products (MRPs) induced new antioxidant properties. Although the concentration of natural antioxidants was reduced, the overall antioxidant properties of food products were maintained or even enhanced.

Anthocyanins, the unique compounds in colored wheat are relatively unstable and often undergo degradation during processing and storage (Jing et al., 2007). Abdel-Aal and Hucl (2003) investigated the stability of anthocyanins at different temperatures and pH. They found that the degradation increased when temperature increased from 65 to 95 ^{o}C and anthocyanins were more thermally stable at pH 1 compared to pH 3 or 5. In addition, Li et al. (2007) reported that no obvious degradation of anthocyanins was observed in heat-treated (177 ^{o}C for 20min) purple wheat bran, whereas heat processing during muffin production (177 ^{o}C for 7-12 min) led to complete destruction of anthocyanins from both heat-treated and non-heat treated purple wheat bran. Sun-Waterhouse et al. (2011) examined the effects of bread-making process on the changes in phenolic compositions of white wheat flours which contained blackcurrant polyphenol extracts composed of flavonoids, phenolic acid and anthocyanins. Baking caused oxidation of quercetin and myricetin leading to a loss of about 61-70 % of polyphenols (Sun-Waterhouse et al., 2011). So far, the changes of phenolic compounds during bread-making have not been well documented and further investigation are needed to specify the effect of each bread-making step on phenolic changes and total antioxidant activity.

1.4. *Ex-vivo* Evaluation of Antioxidant Activity

1.4.1. In-vitro Analysis of Antioxidant Activity Assays

A variety of *in-vitro* chemical methods are used to determine the antioxidant activity of phenolic extracts. The typical assays include total phenolic content (TPC), total antioxidant activity (TAA), ferric reducing/antioxidant power (FRAP) assay, DPPH radical scavenging capacity assay, oxygen radical absorbance capacity (ORAC), total radical absorption potential (TRAP) and trolox equivalent antioxidant capacity (TEAC).

TPC estimates the antioxidant activity of a substance by measuring its capacity to reduce Folin-Ciocalteu reagent, which changes the color from yellow to dark blue (Moore and Yu, 2008). TAA measures the antioxidant capacity of a biomolecule via a single electron transfer (SET) mechanism (Ou et al., 2002) which involves transfer of a single electron from the antioxidant molecule to the oxidative agent (copper II). FRAP is devised to simulate the environment of plasma (Collins, 2005) and is a colorimetric method, in which a colored ferric complex (Fe III) is reduced to its ferrous form (Fe II). In FRAP, antioxidant capacity can also be measured using iron (II) chelating and copper (II) chelating capacity assays, which compete with synthetic chelator to form chelating complexes (Moore and Yu, 2008). DPPH assay involves direct reaction of antioxidants with DPPH radicals, which causes change in absorbance and is monitored spectrophotometrically. ORAC, TRAP and TEAC assays utilize competitive kinetics to compare the ability of antioxidants and fluorescent probe in scavenging free radicals. Antioxidant capacity is obtained by measuring the fluorescence decay of the probe in relation to a control (Collin, 2005; Ou et al., 2002).

1.4.2. Ex-vivo Analysis of Antioxidant Activity Assays

1.4.2.1. Cell viability assays

The effects of phenolic compounds on oxidative stress are often investigated using cell culture assays. Theoretically, the measurement of oxidative stress can be estimated directly by the amount of reactive species present in the cells (McKenna, 2009). However, the short survival time of reactive species makes the direct assessment difficult to perform (Halliwell and Whiteman, 2004) except when some techniques such as L-band electron spin resonance and magnetic resonance imaging spin are used (Utsumi and Yamada, 2003; Berliner et al., 2001). Oxidative stress can be measured indirectly by assessing cell viability (McKenna, 2009). Trypan blue dye exclusion is an example of such indirect measurement. This assay is based on the principle that living cells have the ability to take up the dye while dead cells cannot. Therefore, their cell membranes appear visible because they are no longer able to control the passage of macromolecules (Stoddart, 2011). Other cell viability measurement methods include resazurin-based methods, the measurement of up-taking radioactive labeled H-thymidine into cellular DNA, and the reduction of different kinds of tetrazolium salts (Stoddart, 2011; Yin et al., 2013). Among these assays, MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazo-lium bromide), which is a kind of tetrazolium salt, is widely used. This method is based on the principle that the metabolically active cells reduce the yellow MTT reagent to an insoluble purple formazan dye crystal. After dissolving these crystals into solution, the absorbance can be measured spectrophotometrically.

1.4.2.2. Cellular antioxidant activity assay

As a fluorescent probe, dichlorofluorescin diacetate (DCFH-DA) was first used by

Keston and Brandt (1965) to quantify hydrogen peroxide in cell-free system. Bass et al. (1983) later modified this method and used it in cultured cells to measure the respiratory burst hydrogen peroxide in phobol myristate acetate (PMA)-stimulated polymorphonuclear leukocytes. Recently, the cellular antioxidant activity (CAA) was developed by Wolfe et al. (2007) to evaluate the antioxidant capacity of dietary supplements, phytochemicals and food components. The mechanism of this method is summarized in **Figure 1.6**.

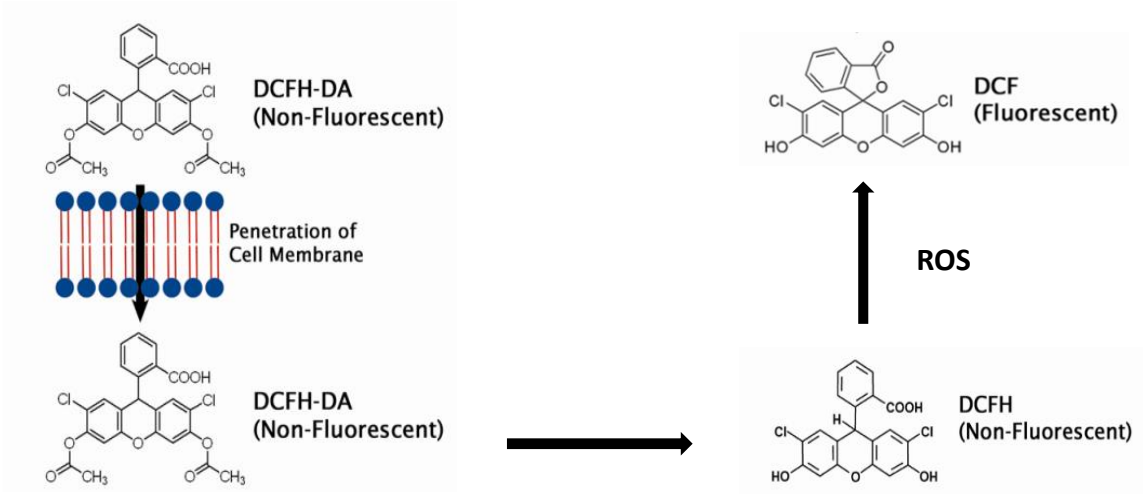


Figure 1.6 Mechanism of cellular antioxidant activity assay

In CAA assay, cells are cultured in a 96-well plate and then incubated with DCFH-DA. When diffused into the cells, DCFH-DA is deacetylated by cellular esterase to a non-fluorescent substance - DCFH. In the presence of reactive oxygen species (ROS), DCFH will be oxidized to DCH, which is highly fluorescent. The intensity of fluorescence is proportional to the level of ROS within the cell. The addition of antioxidants prevents oxidation of DCF, therefore, decreases the fluorescence intensity. By comparing with predetermined DCF standard curve, the antioxidant activity can be measured. This assay is

considered more biologically relevant than the chemical antioxidant activity assays (Song et al., 2010), because it accounts for some aspects of uptake, metabolism and location of antioxidant compounds within the cells (Wolfe et al., 2007).

1.4.2.3. Cell lines

Primary cell culture involves growing cells in a favorable artificial environment. Cells that are isolated directly from a tissue are known as primary cells (Freshney, 2006). Typically, primary cells are composed of a mixture of cell types and proliferated under the appropriate conditions. When they reach confluence, the primary cells have to be sub-cultured into another vessel with fresh growth medium (Freshney, 2006). Cell lines come from primary cells that have been transformed by various means and immortalized. Normally, cell lines have a finite life span (can divide only a limited number of times) because of cell senescence (Freshney, 2006). However, some cells, such as tumor cells are inherently immortal and other cells may become immortal through suffering a process called transformation (Freshney, 2010). Cell transformation can occur spontaneously or induced by chemicals or virus (Freshney, 2010). These transformed cells normally have high growth capacity, therefore predominating in the population and causing a genotypic and phenotypic uniformity. The continuous cell lines have the ability to divide indefinitely under favorable conditions.

The accurate *ex-vivo* assessment of phenolic compounds is based on the proper selection of cell lines. Various cell lines have been utilized to study cereal polyphenols as summarized in **Table 1.1**.

Table 1.1 Studies on phenolic compounds of cereal grains using cell culture models

Tested sample	Cell lines	Purpose	Reference
Ferulic acid antioxidants	Hippocampal neuronal cells	Effects on neurodegenerative disorders such as Alzheimer's disease	Kanski et al., 2002
Rice pigment extracts	Murine macrophage RAW 264.7 cells	Suppressing effects on ROS and RNS	Hu et al., 2003
Avenanthramides, a phenolic antioxidant present in oats	Human aortic endothelial cell (HAEC) monolayers	Anti-inflammatory and antiatherogenic effects of avenanthramides	Liu et al., 2004
Water-soluble feruloyl oligosaccharides from wheat bran insoluble dietary fibre	Rat erythrocyte	Inhibitory effect on rat erythrocyte hemolysis	Yuan et al., 2005
Avenanthramide, a polyphenol from oats	A10 cell line derived from rat embryonic aortic smooth muscle cells	Effect of avenanthramide on proliferation of vascular smooth muscle cells and impaired nitric oxide production	Nie et al., 2006
Phenolic compounds from rice grain varieties	Rat hepatoma H4IIE cell line	Protective effect of ethanolic rice extracts on oxidative stress and apoptotic cell death	Chi et al., 2007
Anthocyanin extracts from blue wheat	Mouse macrophage RAW264.7 cells	Suppression activity of pigmented wheat on ROS and RNS activity	Hu et al., 2007
Extracts from wheat aleurone	Human colon adenocarcinoma cell lines HT-29	Inhibitory effects on human HT29 colon adenocarcinoma cells.	Borowicki et al., 2010
Phenolic extracts from purple wheat infant cereals	Primary human epithelial cell line FHs 74 Int obtained from the small intestine of a human fetus	Cellular antioxidant activity of phenolic compounds in infant cereals	Hirawan et al., 2011
Dietary fibre phenolic compounds	Human colon adenocarcinoma cell lines Caco-2	Antiproliferative effect on the cell cycle of Caco-2 cells	Janicke et al., 2011
Free and bound cowpea phenolics	Hormone-dependent mammary (MCF-7) cancer cells	Inhibitory effect of cowpea phenolics on cell proliferation of mammary cancer cells	Gutierrez-Uribe, et al., 2011
Wheat antioxidants	Human colorectal adenocarcinoma cell lines HT-29 and Caco-2	Antiproliferative activities of wheat antioxidant to cancer cells	Lv et al., 2012
A flavone, tricin extracts from winter wheat hull	Rat pancreatic cells line (INS832/13) and Human hepatocellular carcinoma cells (HepG2)	Inhibitory effects on inhibitor of two cancer cell lines of liver and pancreas	Moheb et al., 2013

The use of appropriate cell line models is crucial for the evaluation of properties of antioxidants including their effects on cell proliferation, apoptosis and cancer progression. Examples of commercial cell lines have been summarized in **Table 1.2**.

Table 1.2 Examples of some widely used cell lines with origin in different cell types

Cell lines	Origins
Colorectal cell lines: derived from colon tumors	
[a]CACO-2	Human Caucasian colon adenocarcinoma
[a]HT29	Human Caucasian colon adenocarcinoma
Neurobiology cell lines:	
[a]SK-N-DZ	Human neuroblastoma
[a]N1E-115 Mouse neuroblastoma	N1E-115 Mouse neuroblastoma
Oesophageal cell lines: derived from oesophageal adenocarcinoma	
[a]FLO-1	Distal oesophageal adenocarcinoma
[a]OE19	Human Caucasian oesophageal carcinoma
[a]ESO26	Adenocarcinoma of the gastroesophageal junction
[a]SK-GT-2	Adenocarcinoma of the gastric fundus, poorly differentiated
[a]SK-GT-4	Oesophagus adenocarcinoma, well-differentiated
[a]OACP4 C	Gastric cardia adenocarcinoma
Small intestine cell lines:	
[a]IEC 6	Rat small intestine epithelium
[b]Ca Ski	Human small intestine and cervix

[a]Information was obtained from the European Collection of Cell Cultures (ECCC)
[b]Information was obtained from American Type Culture Collection (ATCC)

Among the various cell lines available for analysis, the human colorectal cell lines and small intestinal cell lines were employed in the present study because of the metabolic pathways of cereal phenolic compounds. Free phenolic compounds are absorbed into the human circulatory system (Andersson et al., 2010) through the small intestine (Vitaglione et al., 2008). On the other hand, majority of phenolic compounds are esterified and bound to cell well polysaccharides, and therefore require release by intestinal enzymes or by colon microflora fermentation (Andersson et al., 2010). During this digestion process, phenolics help to quench the free radicals that are continuously formed in the intestinal tract. Therefore,

human small intestinal cell lines and colorectal cell lines may be a better choice because small intestine and colon are the major sites where phenolics perform their functions.

1.5. Factors Leading to Pro-oxidant Properties of Phenolics in *ex-vivo* Analysis

The antioxidant activity and disease-preventive effects have been well established both *in-vitro* and *ex-vivo* (Halliwell, 2008). However, polyphenolic compounds can also exert pro-oxidant effects under certain experimental conditions (Prochazkova et al., 2011). Song et al. (2010) pointed out that the results from CAA (*ex-vivo*) significantly correlated to total phenolic content (*in-vitro*) but were less correlated to the ORAC values (Wolfe et al., 2008). Three factors, namely metabolites of phenolic compounds, concentration dependent manner and excessive oxidative stress in cells, are discussed in an attempt to unveil the ambiguity of various findings.

1.5.1. Metabolites of Phenolic Compounds in Cells

As extracellular substances, some polyphenols are decomposed by cellular metabolism, and antioxidant functional groups are converted to lower or non-antioxidant compounds. For example, the cellular o-methylation and glucuronidation reduce the antioxidant capacities of quercetin, (−)-epicatechin, catechin and luteolin (Prochazkova et al., 2011). Thus, in some studies, the metabolism of polyphenols is attributed to their cellular pro-oxidant properties (Metodiewa et al., 1999; Bayrakceken et al., 2003; Tu et al., 2011). For instance, enzymatic oxidation of quercetin would produce o-semiquinone and o-quinone which were responsible for facilitating the formation of superoxide and depletion of glutathione (GSH) (Metodiewa et al., 1999). Bayrakceken et al. (2003) indicated that when flavonoids react with ROS in cells,

they would be converted to flavonoid phenoxyl radicals (Fl-O•), which might be subjected to further oxidation to produce flavonoid quinones. Flavonoid quinones were still reactive and were implicated for the pro-oxidant properties of flavonoids (Tu et al., 2011). Evidence also indicated that the phenoxyl radicals of apigenin, naringenin and naringin rapidly oxidized NADH, yielding extensive uptake of oxygen and formation of $O_2^{\bullet -}$ (Galati et al., 2002). Moreover, catechol, luteolin, eriodictyol and quercetin were found to oxidize low molecular weight antioxidants such as ascorbate, NADH and glutathione (GSH) (Prochazkova et al., 2011). The oxidized antioxidants reacted other antioxidants to produce an antioxidant network (Bast et al., 1991) leading to an increase in oxidative stress in the cells.

1.5.2. Concentration Dependent Pro-oxidant Properties

Phenolic pro-oxidant properties were reported to be concentration dependent. Labieniec and Gabryelak (2007) monitored the pro-oxidant effect of tannic, ellagic and gallic acid on digestive gland cells of *Unio tumidus*. The cell apoptosis was attributed to the high concentration of phenolic acids. Similarly, Yen et al. (2003) also investigated the pro-oxidant properties of quercetin, naringenin, hesperetin and morin. As the concentration of each flavonoid increased, the generation of superoxide anion radicals and products of lipid peroxidation increased in human lymphocytes. Some phenolic compounds exert both antioxidant and pro-oxidant properties depending on the concentration used. For example, quercetin and myricetin were found to be powerful antioxidant to inhibit lipid oxidation at low concentration (1.5uM), while the hydroxyl radical formation increased 8 times when high concentration (100uM) was used (Laughton et al., 1989). In addition, 10 uM quercetin had no

significant effect on cell viability until the concentration increased to 200 uM (Metodiewa et al., 1999). Furthermore, quercetin was effective in reducing the level of superoxide-induced oxidation of DNA damage at low concentration (~50uM). However, when the concentration increased to 100uM, the extent of damage increased inversely (Wilms et al., 2008).

1.5.3. Excessive Oxidative Stress in Cell Culture

Based on the principle of CAA, known amounts of reactive species (H_2O_2, AAPH•, ABAP etc.) are introduced into the cells. However, other than the introduced radicals, cell culture itself imposes oxidative stress. The CAA is based on evaluation of generated oxidative stress rather than a particular reactive species (Halliwell and Whiteman, 2004). Therefore, the oxidative stress produced during CAA may lead to misinterpretation of the results. Studies have shown that oxidation of DCFH by various radicals is likely to produce intermediate radicals which interact with intrinsic cellular substances, such as NADH and GHS, to produce more free radicals (Rota et al., 1999). Moreover, the condition of cell culture (95% air/5% CO_2) is a state of hyperoxia (1-10 mm Hg in human body vs. 150 mm Hg in cell culture models), which increases the production of reactive oxygen species by cellular enzymes or leakage from electron transport chains (Halliwell, 2008). This excessive oxidative stress may lead to inaccurate measurement. For instance, the fluorescent intensity induced by intrinsic oxidative stress could be assumed to contribute to the pro-oxidant properties of tested samples.

CHAPTER II. Identification and Antioxidant Properties of Phenolic Compounds during Production of Bread from Purple Wheat Grains

2.1. Abstract

Phenolic profiles and antioxidant properties of purple wheat varieties were investigated to document the effects of the bread-making process. Bread crust and crumb along with samples collected after mixing, 30 min fermenting, 65 min fermenting and baking were examined. Phenolic contents including soluble and insoluble phenolic compounds as well as total anthocyanins were determined. 2,2'-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization capacity were employed to determine their antioxidant properties. HPLC analysis was used to detect the presence of soluble, insoluble phenolic acids and anthocyanins. Free phenolic content (105.4~113.2 mg FAE/100g) significantly ($p<0.05$) increased during mixing, fermenting and baking (65~68%). Bound phenolics slightly ($p>0.05$) decreased after 30 min fermentation (7~9%) compared to the dough after mixing, but increased significantly ($p<0.05$) during 65 min fermenting and baking (16~27%). Their antioxidant activities followed a similar trend as observed for total phenolic content (TPC). The bread crust demonstrated increased free (103~109%) but decreased bound (2~3%) phenolic content, whereas bread crumb exhibited a reversal of these results. Total anthocyanin content (TAC) significantly ($p<0.05$) decreased by 21% after mixing; however, it gradually increased to 90% of the original levels after fermenting. Baking significantly ($p<0.05$) decreased TAC by 55% resulting in a lowest value for bread crust (0.8 to 4.4 mg cyn-3-glu equiv./100g). *p*-Hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids were detected in free-

while protocatechuic, caffeic syringic and sinapic were additional acids in bound-phenolic extracts. Cyanidin-3-glucoside was the detectable anthocyanin in purple wheat. The bread-making process significantly ($p<0.05$) increased the phenolic content and antioxidant activities; however, it compromised the anthocyanin content of purple wheat bread.

2.2. Introduction

Wheat as one of the staple foods worldwide is not only regarded a source of protein and carbohydrates, but it is also recognized for its potential in reducing the risk of oxidative-stress related chronic diseases and age-related disorders, such as cardiovascular diseases, neurodegeneration, type II diabetes, obesity and some cancers (Quideau et al., 2011; Okarter et al., 2010). The potential health benefits are partly attributed to its unique phytochemical composition. In recent years, these trace amounts of antioxidant phytochemicals have attracted considerable interest by both researchers and food manufacturers. Phenolic compounds are present in wheat as secondary plant metabolites for normal functions (Challacombe et al., 2012). The most abundant antioxidants present in wheat are phenolic acids (Beta et al, 2005). The substances that contribute to the distinction of pigmented wheat and common wheat are anthocyanins. Anthocyanins, contribute significantly to the antioxidant activity of colored wheat (Liu et al., 2010).

Generally, whole wheat as harvested is not ingested directly by humans but requires some processing prior to consumption. Bread as one of the processed cereals made from wheat is an important food product in human diets, and therefore acts as a suitable carrier for health-promoting compounds. There is growing consumer interest in ingesting whole-meal

products because of the concentrated dietary fibre and phytochemicals in the outer layers of the grains (Parker et al., 2005; Katina et al., 2005). Even though increasing evidence has indicated the health potential of wheat, food manufacturing may compromise its functional properties, resulting in loss of antioxidant activity. There is substantial literature on the *in-vitro* antioxidant properties of phenolic acids and anthocyanins. However, their specific changes at each critical step of bread-making process have not been adequately investigated. Therefore, the present research aimed to investigate the changes of phenolic acids and anthocyanins as well as their antioxidant activities during bread production.

2.3. Materials and Methods

2.3.1. Chemicals and Standards

Analytical grade acetic acid, Folin–Ciocalteu reagent, 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox) and phenolic acid standards (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, sinapic acid) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). HPLC grade methanol and ethyl acetate were purchased from Fisher Scientific Co. (Ottawa, ON). Deionized water (Milli-Q) was used in HPLC analysis.

2.3.2. Sample Description

Three bread wheat grains (*Triticum aestivum*) were received from Mørdrupvej 5 DK-3540 Lynge (Lynge, Denmark), including Indigo (97% purple kernels), Konini (84% purple kernels) and Öelands hvede (yellow kernels). These three wheat grains were all spring wheats

grown in 2012 at Mørdrupård, 30 km northeastern from Copenhagen, Denmark from a certified organic farm (latitude N 55°49.530′, longitude E 012°13.587′). The grains were milled using a Häusler Mühle model HS with a 50 cm Naxos stone (Häussler, Karl-Heinz Häussler GmbH, Albstadt, Germany).

Indigo Konini Öelands hvede

2.3.3 Flour Composition Analysis

The moisture contents of whole wheat flour were determined using AACC International Approved Method 44-15.02. Total ash contents of whole wheat flour were determined according to AACC International Approved Method 08-01.01. All samples were analyzed in duplicate.

2.3.4. Bread-making Procedure

Bread was baked according to Canadian short process method (CSP method, Canadian Grain Commission) with some modifications. The formula included 100 g flour (14% moisture basis), 3 g compressed yeast, 2.5 g gluten, 4 g whey powder, 4 g sugar, 2.4 g salt, 1 g malt, and 3 g pure lard. The farinograph absorption ((FAB) was determined by a Brabander DoCorder model 2200-3 （Brabender Instruments, Inc, South Hackensack, NJ), which was modified by the Grain Research Lab (GRL, Winnipeg, MB, Canada). The amount of liquid

added was calculated as: (FAB – 3 mL) water (37 °C) + 1 mL ammonium phosphate solution (0.1%). Mixing took place in a pin mixing bowl (100 g Mixer, National Manufacture Co., Lincoln, NE, USA) at 140 rpm and 30 °C. Optimum mixing time was 10% past peak consistency. The dough was fermented in a warm cabinet (12 door-proofer with humidity and temperature controller) at 37.5 °C and 85% relative humidity. After 15 min, the dough was punched lightly 7 times by hand. Afterwards, the dough was placed back into the warm proofing cabinet for another 15 min and subsequently was processed through a sheeter (GRL manufactured sheeter, Canadian Grain Commission) at 11/32 and 3/16 levels, respectively. The dough was molded immediately using a GRL molder for 30 s. The bread dough was proofed for 35 min, which was determined by maximum height. The bread baking oven (Model 6 Precision Scientific, National Manufacturing Company, Lincoln NE, U.S.A.) was preheated to a set temperature of 200 °C. The dough was baked for 25 min. To avoid the introduction of antioxidants, pure lard was used instead of shortening. Each sample was produced in two batches and the average value was reported.

2.3.5. Sample Preparation

At the end of mixing, 30 min fermenting, 65 min fermenting and baking, samples were removed immediately into -20^{o} C freezer to terminate the chemical changes. All the samples were freeze dried along with the raw flour, bread crust (5 mm thick outside the whole bread) and crumb (crust removed bread inner-side) and ground to pass through a 0.42 mm sieve. All the samples were stored at 4 °C before analysis.

2.3.6. Extraction of Soluble Phenolic Compounds

Finely ground sample (2 g) was extracted twice with 80% methanol at a ratio of 1:5 (w/v) (totally 20 mL of 80% methanol). Each time, the mixture was shaken for 1.5 h using a wrist action shaker (Burrell, Pittsburgh, PA) and then sonicated for 0.5h (Branson 5510, Richmond, VA) under nitrogen and dark condition at ambient temperature. The mixture was centrifuged at 11,963 g and 4 °C for 15 min (Sorvall RC 6+, Thermo Fisher Scientific, Ottawa, Ontario). The supernatants were combined and filtered with a φ 90mm filter paper (Whatman™ Cat No. 1004 125). The collected crude extracts were stored at -20 °C until the analysis for free phenolic content, DPPH• scavenging activity and $ABTS^{\bullet+}$ decolorization capacity. To identify and quantify the soluble phenolic acids, the crude extracts were filtered with 0.45 μm syringe filters (VWR International, Cat No. 28146-489) prior to the injection into HPLC. The residues were dried in a fume hood at room temperature and kept in sealed bags at 4 °C before alkaline hydrolysis. Extraction was done in duplicate.

2.3.7. Extraction of Insoluble Phenolic Compounds

The residue collected after 80% methanol extraction was subjected to alkaline hydrolysis. Insoluble phenolic compounds were released using the method described by Qiu et al. (2010) with some modifications. First, 15 mL of 4M NaOH was added to the dried residue (0.5 g) and hydrolyzed on a shaking water bath (VWR, Radnor, PA, USA) for 4 h under N_2 and at 250 rpm and 25 °C. The hydrolyzed mixture was adjusted to a pH between 1.5 to 2.0 with 6 N HCl and then extracted three times with ethyl acetate (25mL × 3). Every time after mixing, ethyl acetate was separated from the aqueous layer by centrifuging at 11,963 g and 4 °C for 5 min (Sorvall RC 6+, Thermo Fisher Scientific, Ottawa, Ontario). The supernatant was

collected into an Erlenmeyer flask using a pipette. Further dehydration was performed by adding 1 g of Na_2SO_4 and then filtered with a filter paper (φ 90mm, Whatman™ Cat No. 1004 125). The combined ethyl acetate extracts were evaporated and dried at 35 °C under vacuum using a rotary evaporator (Yamato RE-51, Cole-Parmer Instrument Company, IL, USA) with a water bath (Thermo-Lift, Fisher Scientific, NJ, USA). The dried residue was redissolved in 2 mL of 50% methanol and stored at -20 °C. The extract was used for the determination of bound phenolic content, DPPH• scavenging activity and $ABTS^{\bullet+}$ decolorization capacity. To investigate the presence of insoluble phenolic acids, HPLC analysis was also performed. The samples were filtered with 0.45 μm syringe filters (VWR International, Cat No. 28146-489) before injection. Extraction was done in duplicate.

2.3.8. Extraction of Anthocyanins

Extraction of anthocyanins was performed according to a method summarized by Young and Abdel-Aal (2010) with modifications. Specifically, finely ground sample (1g) was extracted with acidified methanol/HCl (1 N) (85:15, v/v) at a ratio of 1:8 (w/v). The pH of the mixture was adjusted to 1. The mixture was shaken for 1.5 h (Wrist action shaker, Burrell, Pittsburgh, PA), followed by sonication for 0.5 h (Branson 5510, Richmond, VA) under nitrogen and in the dark at ambient temperature. The mixture was then centrifuged at 11,963 g and 4 °C for 15 min (Sorvall RC 6+, Thermo Fisher Scientific, Ottawa, Ontario). The supernatant was filtered with a φ90mm filter paper (Whatman™ Cat No. 1004 125) and evaporated to dryness at 35 °C under vacuum using a rotary evaporator (Yamato RE-51, Cole-Parmer Instrument Company, IL, USA) with a water bath (Thermo-Lift, Fisher Scientific, NJ, USA). The residue

was then redissolved in 2 mL of 80% methanol. The crude extracts were kept at -20 °C for 2 days to precipitate large molecules and then filtered through a 0.45 um syringe filter (VWR International, Cat No. 28146-489). The extracts were stored at -20 °C prior to the determination of total anthocyanin content (TAC) with pH differential method, DPPH• scavenging activity and $ABTS^{\bullet+}$ decolorization capacity. Extraction was done in duplicate.

2.3.9. Determination of Phenolic Content

The free, bound and total phenolic contents (FPC, BPC and TPC) were determined using a Folin-Ciocalteau method as described by Li et al (2007). Briefly, 10 fold dilution of Folin-Ciocalteu reagent was prepared just prior to use. Then 1.5 mL of freshly diluted Folin-Ciocalteau reagent was used to oxidize 0.2 mL sample extracts. After allowing the mixture to equilibrate for 5 min, the reaction was then neutralized with 1.5 mL sodium carbonate solution (60g/L) at room temperature. The absorbance of the resulting solution was measured at 725 nm after 90 min against a blank of 80% or 50% methanol (80% methanol for soluble phenolic extracts and anthocyanin extracts, 50% methanol for insoluble phenolic extracts). Ferulic acid (FA) and gallic acid (GA) were used as standards. Therefore, free, bound and total phenolic content of samples were expressed as mg of FAE (equivalents)/100g and mg of GAE/100g.

2.3.10. DPPH Radical Scavenging Capacity Activity Assay

The DPPH method was used according to the modified method used by Beta et al. (2005). A 60 μmol/L DPPH• reactant was made in methanol. Then 3.9 mL of DPPH• solution was added to 0.1 mL of sample and the absorbance at 515 nm was measured at t = 30 min. To

determine the absorbance at t = 0 min, measurement was taken by adding 3.9 mL of DPPH• solution to 0.1 mL of 80% or 50% methanol (80% methanol for soluble phenolic extracts and anthocyanin extracts, 50% methanol for insoluble phenolic extracts). The antioxidant activity was calculated as: % DPPH• scavenging activity = (1 - [$A_{\text{sample},t=30}$ / $A_{\text{control},t=0}$]) × 100. A plot of trolox concentration versus % DPPH• scavenging activity was used as a standard curve. DPPH value was expressed as umol TE (Trolox equivalents)/100g.

2.3.11. ABTS Radical Cation Decolorization Assay

The ABTS$^{\bullet+}$ reagent was made by mixing ABTS$^{\bullet+}$ stock solution (7 mM) (38.4mg ABTS in 10 ml distilled water) and potassium persulfate stock solution (2.45 mM) (6.62mg potassium persulfate in 10 ml distilled water) at a ratio of 1:1 for 2 minutes vortex. The mixture was stored for 12 to 16 hrs in the dark at room temperature prior to use. Then, 5 mL of ABTS$^{\bullet+}$ reagent was diluted with approximately 495 mL of distilled water and absorbance at a wavelength of 734 nm was adjusted to 0.7 by diluting the solution further with distilled water. The antioxidant activity of soluble, insoluble phenolic extracts and anthocyanin extracts (50 µL) were evaluated by adding 1.85 mL of freshly made ABTS$^{\bullet+}$ reagent. The absorbance was determined at t = 30 min. For absorbance at t = 0 min, 1.85 mL of freshly made ABTS$^{\bullet+}$ reagent was added to 50 µL of 80% or 50% methanol (80% methanol for soluble phenolic extracts and anthocyanin extracts, 50% methanol for insoluble phenolic extracts). The ABTS $^{\bullet+}$ decolorization (%) was calculated as (1 - [$A_{\text{sample},\ t=30}$ / $A_{\text{control},t=0}$]) × 100. A standard curve was generated based on different trolox concentrations versus % ABTS$^{\bullet+}$ decolorization and the antioxidant activities of extracts were expressed as umol TE/100g sample.

2.3.12. Absorbance Spectra of Anthocyanin Extract at Different Wavelengths

Finely ground sample (1 g) was extracted with acidified methanol/HCl (1 N) (85:15, v/v) at a ratio of 1:8 (w/v). The mixture was shaken for 0.5 h (Wrist action shaker, Burrell, Pittsburgh, PA) under nitrogen and dark condition at ambient temperature. The mixture was then centrifuged at 11,963 g and 4 °C for 15 min (Sorvall RC 6+, Thermo Fisher Scientific, Ottawa, Ontario). The supernatant was filtered with a φ90mm filter paper (Whatman™ Cat No. 1004 125). The absorbance of the collected supernatant was scanned at wavelengths from 230 to 700 nm using an Ultrospec 1100 Pro UV/Visible spectrophotometer (Amersham Biosciences, USA) controlled by Biochrom Data Capture Software (version 2.0) (Biochrom Ltd. Cambridge, UK). A plot of absorbance versus wavelength was created.

2.3.13. Direct Measurement of Total Anthocyanin Content

Total anthocyanin content was measured according to the method reported by Liu et al. (2010) with some modifications. Briefly, 8 mL of acidified methanol/HCl (1 N) (85:15, v/v) with pH = 1 was added to 1 g of finely ground sample. The mixture was shaken for 1.5 h (Wrist action shaker, Burrell, Pittsburgh, PA), followed by sonication for 0.5 h (Branson 5510, Richmond, VA) under nitrogen and in the dark at ambient temperature. The mixture was centrifuged at 11,963 g and 4 °C for 15 min (Sorvall RC 6+, Thermo Fisher Scientific, Ottawa, Ontario). The supernatant was filtered with a φ90mm filter paper (Whatman™ Cat No. 1004 125). The absorbance at 535 nm was measured without further dilution of the crude extracts. Cyanidin-3-glucoside was used as a standard, which was the primary anthocyanin in wheat (Abdel-Aal et al., 2006). Total anthocyanin content of each sample was calculated as:

$$\frac{A \times Vol \times MW \times 10^2}{\varepsilon \times Sample\ Wt}$$

Where: A is absorbance reading; MW is molecular weight of cyanidin-3-glucoside (449.2 g/mol); ε is molar absorptivity of cyanidin-3-glucoside (25965/cm/M); Wt is dry weight of the ground grain sample. The results were expressed as mg of C3GE (cyanidin-3-glucoside equivalents)/100g sample.

2.3.14. Total Anthocyanin Content using pH Differential Method (TAC)

A second procedure for determination of total anthocyanin content is based on a pH differential method (Lee et al., 2005). Theoretically, anthocyanin pigments undergo reversibly structural transformations when pH changes. This leads to a color change, which can be monitored by spectrophotometer (colored at pH = 1.0, colorless at pH = 4.5). To conduct this assay, two buffer solutions were prepared: one for pH = 1.0 potassium chloride buffer (0.03 M) and the other for pH = 4.5 sodium acetate buffer (0.4 M). Briefly, 1.9 g of KCl was dissolved into 980 mL distilled water and the pH was adjusted to 1 with 6 N HCl, meanwhile, 54.4 g $CH_3CO_2Na \bullet 3H_2O$ was dissolved in 960 mL distilled water and the pH was adjusted to 4.5 with acetic acid. Anthocyanin extracts that had been reconstituted into 2 mL of 80% methanol were used in this assay. Samples were diluted 5 times with both buffers to a final volume of 2 mL, respectively (0.4 mL of anthocyanin extracts in 1.6 mL of either potassium chloride buffer or sodium acetate buffer). The absorbance of each sample was measured at 520 nm against a blank of distilled water after 30 min of preparation. The haze and sediment were corrected by measuring the absorbance again at 700 nm. The concentration (mg/L) of each anthocyanin was calculated according to the following formula and expressed as mg C3GE/100g sample:

$$\frac{A \times MW \times DF \times 10^3}{\varepsilon \times 1}$$

Where: A is the absorbance = $(A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$,

MW is the molecular weight for cyanidin-3-glucoside = 449.2 g/mol,

DF is the dilution factor = 5, L is the pathlength = 1 cm,

ε is the extinction coefficient = 26,900 L $\times$ cm^{-1} $\times$ mol^{-1} for cyanidin-3-glucoside.

2.3.15. Identification and Quantification of Phenolic Compounds with High Performance Liquid Chromatograph

Identification and quantification of phenolic compounds were carried out on an HPLC (Waters 2695, Milford, MA) equipped with a photodiode array (PDA) detector (Waters 996, Milford, MA) and an autosampler (Waters 717 plus, Milford, MA). Analysis was performed on a Gemini 5 μ C18 110 A column (150x4.60 mm) (Phenomenex, Torrance, CA) and the mobile phase consisted of 0.1% acetic acid in water (solvent A) and 0.1% acetic acid in methanol (solvent B). A 70 min linear gradient was programmed as follows: 0-11 min, 9-14% B; 11-14 min, 14-15% B; 14-17 min,15-15% B; 17-24 min, 15-16.5% B; 24-28 min, 16.5-19% B; 28-30 min, 19-25% B; 30-36 min, 25-26% B; 36-38 min, 26-28% B; 38-41 min, 28-35% B; 41-46 min, 35-40% B; 46-48 min, 40-48% B; 48-53 min, 48-53% B; 53-65 min, 53-70% B; 65-66 min, 70-9% B; 66-70 min, 9-9% B. The injection volume was 10 μL and flow rate was 0.9 mL/min. Identification of phenolic acids was achieved by comparison to the retention time of phenolic acid standards and their maximum UV absorption and samples spiked with phenolic acid standards. Phenolic acid quantitation was based on the standard curves of the corresponding phenolic acids at a wavelength of 280 nm and peak area was

used for calculations. The HPLC analyses were done in duplicate for each sample.

2.3.16. Identification of Anthocyanins in Purple Wheat with HPLC-QTOP-MS/MS

The anthocyanin composition was evaluated following a method described by Bicudo et al. (2014). The chromatographic separation was carried out using a HPLC (Waters 2695) system equipped with photodiode array detector (Waters 996) and autosampler (Waters 717 plus) (Waters Corporation, Milford, MA, USA). A Gemini 5 μm RP-18 (150 mm × 4.6 mm) (Phenomenex, Torrance, CA, USA) analytical column was used for separation. Each anthocyanin extract was injected at a volume of 10 μL for analysis. The mobile phase consisted of (A) water with 0.1% formic acid and (B) methanol with 0.1% formic acid at a flow rate of 0.5 mL/min. A linear gradient was programmed as follows: 0-5 min, 5-10% B; 5-15 min, 10-15% B; 15-20 min, 15-20% B; 20-30 min, 20-25% B; 30- 40 min, 25-40% B; 40-45 min, 40-10% B; 45-50 min, 10% B. Anthocyanins were detected at a wavelength of 280 nm. The quadrupole time-of-flight mass spectrometer (Q-TOF-MS) (Micromass, Waters Corp., Milford, MA, USA) was calibrated by using sodium iodide for positive mode through the mass range of 100–1500. MS parameters were set as follows: capillary voltage: 2100 V, sample cone voltage: 30 V, source temperature: 120 ℃, desolvation temperature: 250 ℃, desolvation gas (nitrogen gas) flow rate: 900 l/h. The MS/MS spectra were acquired by using collision energy of 30 V.

2.3.17. Statistical Analysis

The results were reported as mean ± standard deviation (SD) of duplicate determinations.

Analysis of variance (ANOVA) for main factors (flour type, process step and flour type*process step) was determined using GLM procedure with SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). Least significant differences (LSD) were employed to define significant differences among varieties at a level of $p < 0.05$. Pearson correlation test was used to evaluate the correlation among variables at significant levels of $p < 0.05$ and $p < 0.01$.

2.4. Results and Discussion

2.4.1. Flour Composition Analysis

Table 2.1 Moisture, ash and FAB content of raw flours

	Öelands hvede	Indigo	Konini
Moisture content (%) [a]	13.33 ± 0.15	14.96 ± 0.19	14.71 ± 0.08
Ash content (%) [a]	1.78 ± 0.01	1.62 ± 0.00	1.63 ± 0.01
FAB (%) [b]	65.8	63.2	63.8

[a] Results were expressed as mean ± standard deviation (n=2).
[b] FAB stands for farinograph absorption for baking.

Table 2.1 summarizes the moisture, ash and FAB contents for the three bread wheat varieties. Moisture content was determined to convert raw flour onto a 14% moisture basis before formulating the bread. Practically, 99.2 g of Öelands hvede flour, 101.1 g of Indigo flour and 100.9 g of Konini flour were used respectively in the bread-making procedure.

Commercial whole wheat flour is made by mixing a certain amount of wheat bran with refined flour. The large portion of bran in experimental whole wheat flour adds heaviness, therefore preventing the dough from rising. **Picture 2.1** compared the volume of sample bread with the addition of gluten on the left and without the addition on the right. The formed matrix in the left sample held the bread structure better. Therefore, to make bread that would be accepted by consumers, gluten was formulated in the ingredients of bread.

Picture 2.1 Whole wheat bread made with (left) and without (right) the addition of gluten

FAB determined the target water to flour ratio to achieve the maximum dough consistency at a specific speed during mixing.

2.4.2. Free, Bound and Total Phenolic Content using Folin-Ciocalteau Method

Table 2.2 summarizes the amount of soluble free, insoluble bound and total phenolic content at different stages of bread-making process. Results were expressed as mg FAE and GAE/100g. FA was used as a standard because of its predominance among phenolic acids in wheat. Even though GA was rarely detected in wheat, it was still used for a comprehensive comparison among the literature.

Table 2.2 Free, bound and total phenolic content of common and purple wheat grains at different stages of bread-making process

	Free phenolic content (mg FAE/100g)			Bound phenolic content (mg FAE/100g)			Total phenolic content (mg FAE/100g)		
	Öelands hvede^{A}	Indigo^{A}	Konini^{A}	Öelands hvede^{B}	Indigo^{B}	Konini^{B}	Öelands hvede	Indigo	Konini
Flour	113.23±1.97^{kl}	105.44±0.82^{kl}	111.26±0.49^{kl}	125.36±5.26^{efg}	121.82±3.95^{efg}	123.68±5.53^{efg}	238.59±7.24	227.26±4.77	234.94±6.02
Mixing	129.05±1.64^{ghi}	125.76±4.93^{ij}	128.70±0.82^{ghi}	138.57±2.37^{bc}	135.96±2.37^{bcd}	137.08±1.32^{bcd}	267.62±4.01	261.72±7.30	265.78±2.14
30min fermenting	136.14±4.77^{efg}	128±2.47^{hi}	135.67±1.48^{efgh}	129.64±2.37^{cde}	125.73±5.79^{efg}	127.96±2.63^{def}	265.78±7.14	253.73±8.26	263.63±4.11
65min fermenting	142.3±0.66^{e}	130.21±4.28^{fghi}	137.53±6.74^{ef}	141.73±5.26^{b}	138.20±5.53^{bc}	140.61±4.74^{b}	284.03±5.92	268.41±9.80	278.14±11.48
Bread loaf	185.91±3.78^{c}	176.72±0.99^{d}	183.53±2.96^{cd}	158.10±5.79^{a}	154.75±5.79^{a}	156.43±3.95^{a}	344.01±9.57	331.47±6.78	339.96±6.91
Bread crust	228.81±5.92^{a}	219.16±6.08^{b}	229.86±1.81^{a}	122.57±5.00^{efg}	116.80±3.16^{g}	119.59±6.05^{gf}	351.38±10.92	335.96±9.24	349.45±7.86
Bread crumb	123.81±6.08^{ij}	114.28±3.78^{k}	119.05±5.26^{jk}	163.13±0.26^{a}	159.22±4.74^{a}	161.64±8.16^{a}	286.94±6.35	273.50±8.52	280.69±13.42

	Free phenolic content (mg GAE/100g)			Bound phenolic content (mg GAE/100g)			Total phenolic content (mg GAE/100g)		
	Öelands hvede^{C}	Indigo^{C}	Konini^{C}	Öelands hvede^{D}	Indigo^{D}	Konini^{D}	Öelands hvede	Indigo	Konini
Flour	83.15±1.37^{kl}	77.74±0.57^{kl}	81.77±0.34^{kl}	94.32±3.65^{efg}	91.87±2.74^{efg}	93.16±3.83^{efg}	177.47±5.02	169.61±3.31	174.94±4.17
Mixing	94.11±1.14^{ghi}	91.85±1.14^{ij}	93.87±0.57^{efgh}	103.48±1.64^{bc}	101.68±1.64^{bcd}	102.45±0.91^{bcd}	197.60±2.78	193.53±5.06	196.32±1.48
30min fermenting	99.03±3.31^{efg}	93.39±1.71^{hi}	98.71±1.03^{efgh}	97.29±1.64^{cde}	94.58±4.01^{efg}	96.13±1.82^{def}	196.32±4.95	187.97±5.73	194.84±2.85
65min fermenting	103.31±0.46^{e}	94.92±0.46^{fghi}	100.00±4.68^{ef}	105.68±3.65^{b}	103.23±3.83^{bc}	104.90±3.28^{b}	208.98±4.11	198.15±6.80	204.90±7.96
Bread loaf	133.55±2.62^{c}	127.18±2.62^{d}	131.21±2.05^{cd}	117.03±4.01^{a}	114.71±4.01^{a}	115.87±2.74^{a}	250.58±6.64	241.89±4.70	247.08±4.79
Bread crust	163.31±4.11^{a}	156.61±4.11^{b}	164.03±1.25^{a}	92.39±3.47^{efg}	88.39±2.19^{g}	90.32±4.20^{fg}	255.69±7.57	245.00±6.41	254.35±5.45
Bread crumb	90.48±4.22^{ij}	83.87±4.22^{k}	87.18±3.65^{jk}	120.52±0.18^{a}	117.81±3.28^{a}	119.48±5.66^{a}	211.00±4.40	201.68±5.91	206.66±9.31

^{A,B,C,D} Columns labelled with the same capital superscript were considered as a group.

^{a,b,c,d} Significant difference was defined with different letters in each group.

Total phenolic content was calculated as the sum of free and bound phenolic content for each raw.

As seen in **Table 2.2**, FPC of raw wheat flour varied from 113.2, 105.4 to 111.3 mg FAE/100g for Öelands hvede, Indigo and Konini, respectively. The levels were slightly higher than the 100% methanol extracts of black wheat (70.6 to 110.8 mg FAE/100g) reported by Li et al. (2005), but lower than the acidified methanol extracts of colored wheat (146 to 226 mg FAE/100g) reported by Liu et al. (2010). Dough mixing significantly ($p<0.05$) increased FPC by 14.0% to 19.3%. Fermentation also remarkably increased FPC, but its effect was not significant ($p>0.05$) during the first 30 minutes. FPC significantly ($p<0.05$) increased during baking by 64.2% to 67.6%. Bread crust contained the highest FPC, while bread crumb contained a relatively lower FPC. Among the three wheat varieties, Öelands hvede had the highest FPC, whereas the purple wheat, Indigo contained the lowest value. This difference was not significant ($p>0.05$) until the process of 30 min fermentation.

The BPC of raw flour varied from 94.3, 91.9 and 93.2 GAE/100g for Öelands hvede, Indigo and Konini, respectively. The levels were in agreement with the literature reported by Adom et al. (2003), who investigated BPC in whole wheat varieties with a range of 86 to 128 mg GAE/100g, but slightly lower than those reported by Okarter et al. (2010) (93 to 113 mg GAE/100g). Significant ($p<0.05$) increase in BPC occurred during mixing (10.5 ~ 11.6%) and baking (26.1 ~ 27.0%); however, BPC decreased to almost the same level as raw flour after 30 min fermentation. During the next 35 min (65 min fermentation), BPC significantly ($p<0.05$) increased by 13.1 ~ 13.7% compared to raw flour. Unlike FPC, the bread crumb had the highest BPC, while the bread crust contained the lowest value. No significant difference ($p>0.05$) was observed among the three wheat varieties throughout bread-making process.

The major increase in TPC was obtained after mixing and baking. TPC gradually

increased during dough fermentation and the major increase occurred at the last 35 min fermenting step. Bread crust contained the highest TPC, followed by whole bread and bread crumb. These results were in accordance with the findings investigated by Perez-Jimenez et al. (2014), who concluded that bread and bread crust had significantly higher content (Folin-Ciocalteu method) of non-extractable antioxidants than wheat flour. Gelinas and McKinnon (2006) also reported that baking increased the concentration of phenolic compounds while the bread crust contained more phenolic compounds than the crumb.

The functions of intrinsic phenolic antioxidants as structural components in wheat grains have been fully described in the literature. For example, ferulic acid is found ester-bound to arabinoxylans (Garcia-Conesa et al., 1999; Rondini et al., 2004) or ether-linked to lignin or lignin-like polymers (Vaidyanathan and Bunzel, 2012). Phenolic compounds also form complexes with proteins via hydrogen, covalent, ionic bond and hydrophobic interactions (Shahidi and Nacak 1995; Renard et al., 2001; Almajano et al., 2007; Sivam et al., 2010). During mixing, some intrinsic enzymes might be activated upon the addition of water. Along with the mechanical shearing effect, the phenolic complexes might be partially broken down. The increase in FPC was most likely due to the release of some bound phenolic compounds into their free forms. Continuous release of bound phenolic compounds was evidenced by the increase in FPC but decrease in BPC during the first 30 min fermentation. Baking significantly ($p<0.05$) increased the levels of FPC and BPC by approximately 65% and 26%, respectively. This was most likely due to the complex mechanism of baking process, which involved starch gelatinization/pasting, protein denaturation and Maillard reaction (Sivam et al., 2010). These changes occurred simultaneously and possibly led to the release of bound

phenolic compounds. Furthermore, studies showed that the Maillard Reaction Products (MRPs) with reductone type structure or phenol-like complexes exhibited antioxidant activity and were expected to interfere with the absorbance of Folin-Ciocalteu assay (Lee and Shibamoto, 2002; Chandrasekara and Shahidi, 2011). The use of 80% methanol largely incorporated aqueous MRPs into the extract, therefore most likely being detectable in the bread extract. Besides amino acids and reducing sugar, a recent study found that MRPs also formed from phenolic compounds, including phenolic acids and flavonoids (Perez-Jimenez et al., 2014). For example, ferulic acid was incorporated into the melanoidins, contributing significantly to the higher content of bread TPC using Folin assay (Perez-Jimenez et al., 2014).

In terms of bread fractions, bread crust contained the highest FPC followed by whole loaf. Bread crumb contained the lowest value. Nevertheless, BPC was better recovered in bread crumb than crust. This was most likely due to the concentrated MRPs in bread crust. Borrelli et al. (2003) indicated that the lower temperature but higher water activity inside the dough caused the light color of bread crumb leading to the less MRPs in bread crumb.

2.4.3. Antioxidant Activities (AOA) of Soluble and Insoluble Phenolic Compounds

The DPPH• scavenging activities of soluble phenolic extracts are shown in **Figure 2.1a**. The values varied from 139, 127 to 130 μmol TE/100g for Öelands hvede, Indigo and Konini, respectively. The DPPH levels for Öelands hvede, Indigo and Konini significantly ($p<0.05$) decreased by 17%, 21% and 22%, respectively after mixing. This could be explained by the dilution of raw flour with non-antioxidant bread ingredients. The DPPH values recovered to

the initial levels during the first 30 min fermentation, and slightly increased in the next 35 min. Baking significantly ($p<0.05$) increased DPPH• scavenging activity by 85%, 91% and 90% for Öelands hvede, Indigo and Konini, respectively. The increasing effect was better seen in bread crust than crumb. Elevated AOA of soluble phenolic extracts were also reported by Yu et al. (2013) for bread samples. The MRPs – melanoidins have been considered to contribute significantly to the antioxidant properties of baked grain products (Manzocco et al., 2001). No significant ($p>0.05$) difference among wheat varieties was detected in raw flours, during mixing and fermenting. However, significantly ($p<0.05$) lower DPPH level of Indigo was detected after baking.

Figure 2.1b shows the DPPH values for insoluble phenolic compounds. The levels for raw flour were two-fold higher than those of soluble phenolic extracts. On the contrary to soluble phenolic extract, mixing significantly ($p<0.05$) increased the DPPH• scavenging activity except for Indigo flour (increased 13%, 9% and 10% for Öelands hvede, Indigo and Konini). The elevated levels of BPC detected during mixing could contribute to the increase in AOA of insoluble phenolic extracts. Also, the insoluble phenolic compounds might be less sensitive to undergo oxidation than soluble phenolic compounds. During fermentation, slight decrease in DPPH values occurred in the first 30 min and then increased ($p>0.05$) during the next 35 min. Baking significantly ($p<0.05$) elevated the DPPH levels by 47%, 46% and 45% for Öelands hvede, Indigo and Konini, respectively. Unlike DPPH• scavenging activity of soluble phenolic extract, that of insoluble phenolic compounds were considerably higher in bread crumb than in crust. This finding was consistent to BPC in bread, where crust contained the lowest while crumb had the highest BPC. The lower AOA of bread crust could

be attributed to the loss of some phenolic compounds, which were destroyed at high temperature (Han and Koh, 2011).

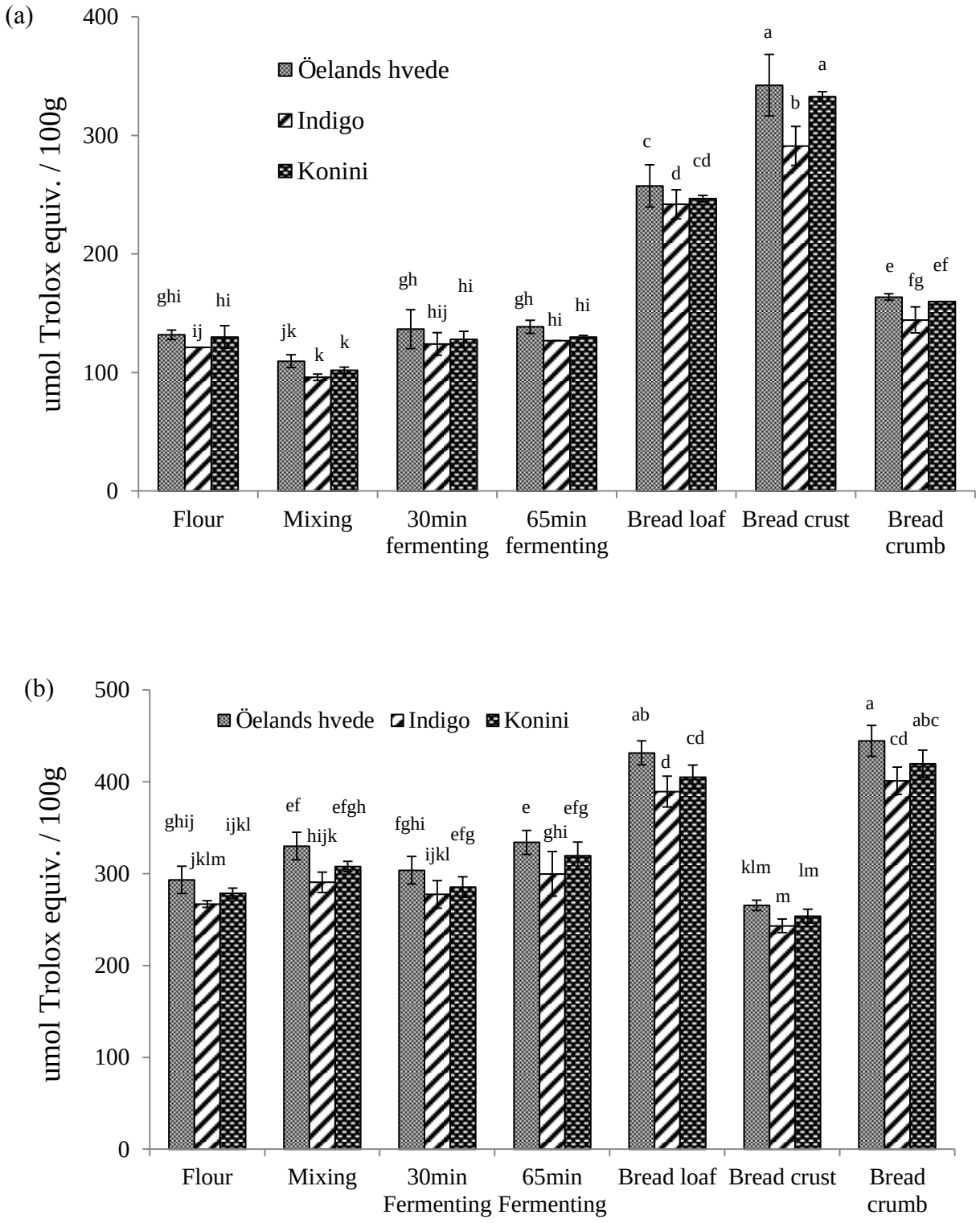


Figure 2.1 DPPH radical scavenging capacity of soluble (a) and insoluble (b) phenolic compounds extract. Columns marked by different letters are significantly different ($p<0.05$).

The $ABTS^{\bullet+}$ scavenging capacity of soluble phenolic extract in raw flour varied from 296, 275 and 283 μmol TE/100g for Öelands hvede, Indigo and Konini, respectively (**Figure 2.2a**). A lower range of 201 to 248 μmol TE/100g for non-colored whole wheat flour was observed by Lv et al. (2014) using 50% acetone. Consistent to DPPH assay, ABTS method detected a significant ($p<0.05$) decrease in AOA during mixing (decreased 42 ~ 46%) and a slight ($p>0.05$) increase during fermentation. Baking significantly ($p<0.05$) increased the AOA levels by 78% to 83% compared to raw flour. Bread crust contained the highest ABTS value (628, 592, 608 μmol TE/100g for Öelands hvede, Indigo and Konini, respectively) followed by whole bread and bread crumb.

The ABTS values of insoluble bound phenolic extract in raw flour varied from 832, 755, 822 μmol TE/100g for Öelands hvede, Indigo and Konini, respectively (**Figure 2.2b**). Similar to findings with DPPH assay, the ABTS values were significantly ($p<0.05$) higher after mixing (increased 11 ~ 16%). Even though significant ($p<0.05$) decrease in AOA occurred during the first 30 min fermentation (decreased 7 ~ 11% compared to mixing dough), the ABTS value increased again during the next 35 min. Baking significantly ($p<0.05$) increased the ABTS value by 32% to 41% compared to raw flour. Bread crumb contained the highest AOA while bread crust possessed the lowest value. Compared to DPPH assay, significant difference was detected during mixing and fermenting using ABTS method. The higher sensibility might be partially due to the solubility and diffusivity of free radicals in organic solvent and/or due to the react ability of native substitutes in extracts, such as phenolic acids, flavonoids, and MRPs, to DPPH and ABTS radicals.

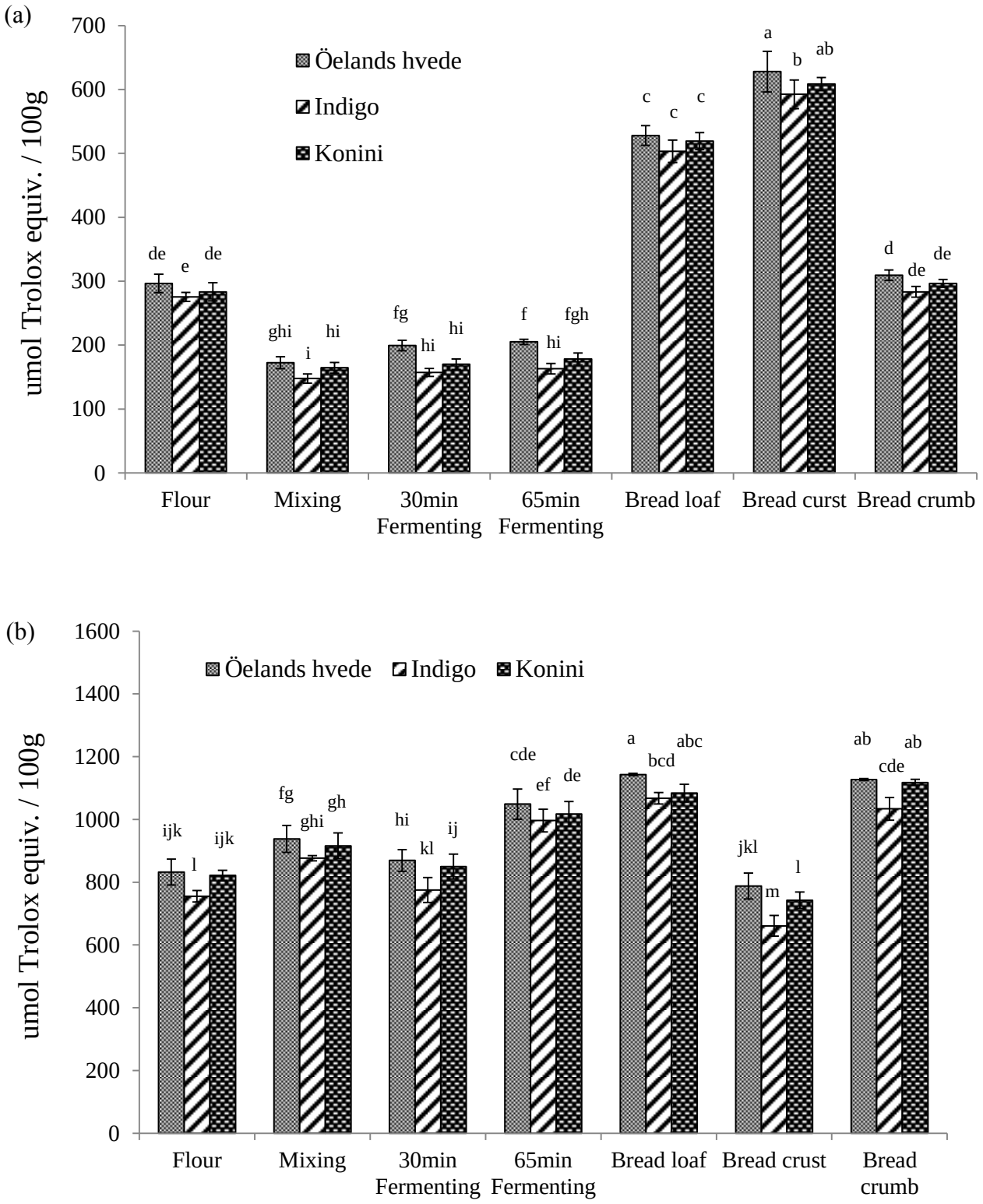


Figure 2.2 ABTS radical cation decolorization activity of soluble (a) and insoluble (b) phenolic compounds extract. Columns marked by different letters are significantly different ($p<0.05$).

2.4.4. Identification and Quantification of Phenolic Acids in Soluble and Insoluble Phenolic Extracts

Monomeric phenolic acids in soluble-free and insoluble-bound phenolic extracts were identified using HPLC by comparing with the retention times and maximum UV absorption of external standards. Sample spiked with external standards was used for further identification. Quantification of monomeric phenolic acids was accomplished on the basis of relating LC chromatogram data to phenolic acid standard curves. These commercial phenolic standards include five hydroxybenzoic acids (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic and syringic acid) and four hydroxycinnamic acids (caffeic, *p*-coumaric, ferulic and sinapic acid). Identification and quantification were performed at a wavelength of 280 nm, which was confirmed to be more capable for detecting all phenolic acids by previous studies of Guo and Beta (2013) and Qui et al. (2010).

Figure 2.3 summarizes the chromatograms of soluble-free and insoluble-bound phenolic extracts of bread made from the three wheat varieties. The detected monomeric phenolic acids were labeled and numbered according to the retention time. In soluble free phenolic extracts, *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acid were detectedwhile other than gallic acid, the other eight phenolic acids were identified in alkaline extracts.

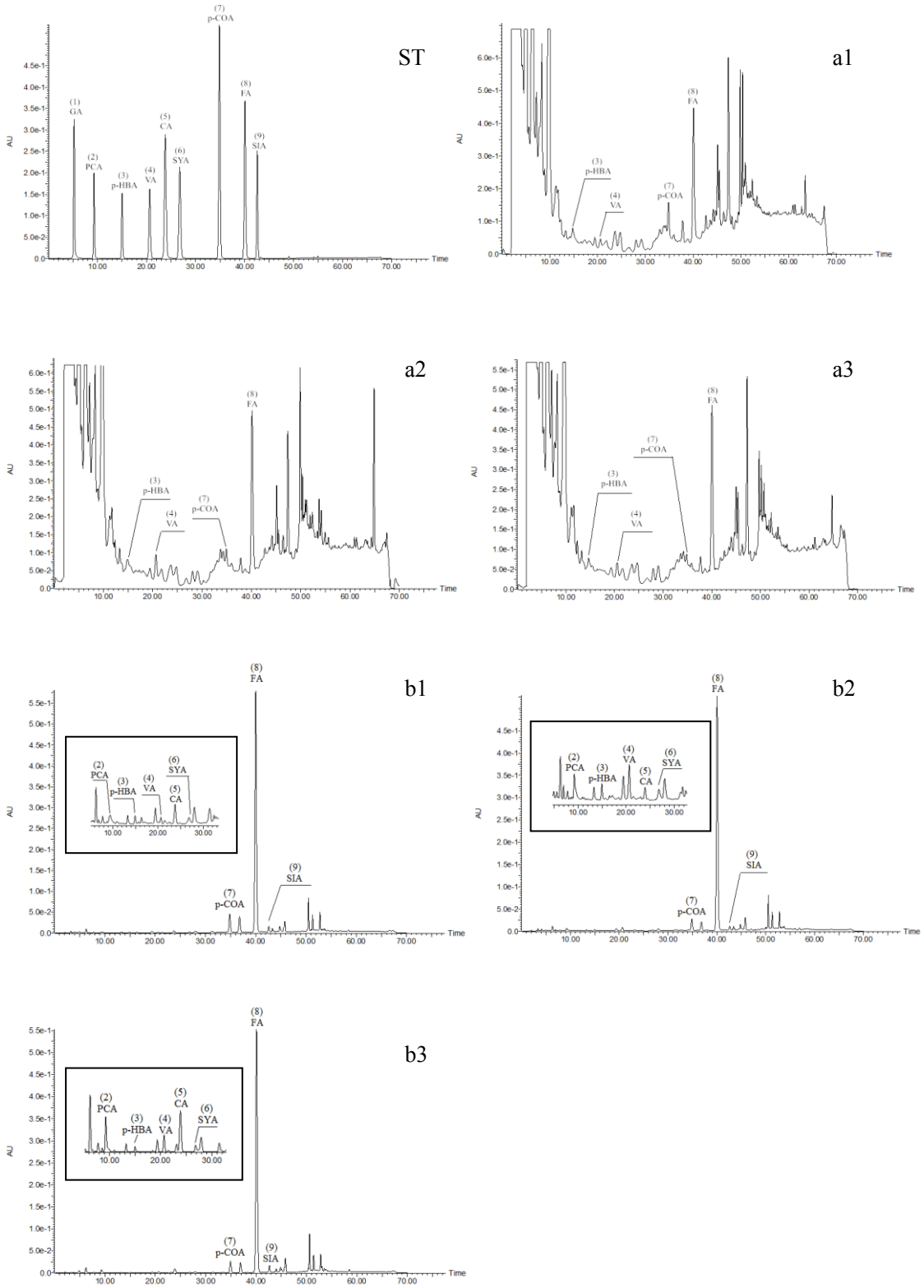


Figure 2.3 HPLC chromatograms of soluble-free (a) and insoluble-bound phenolic extracts (b) from Öelands hvede (1), Indigo (2) and Konini (3) bread samples at 280 nm. ST, phenolic acid standards: (1) gallic acid, (2) protocatechuic acid, (3) *p*-hydroxybenzoic acid, (4) vanillic acid, (5) caffeic acid, (6) syringic acid, (7) *p*-coumaric acid, (8) ferulic acid, (9) sinapic acid

Table 2.3 shows the content of detected monomeric phenolic acids in soluble fraction during bread production. In raw wheat flour, only free ferulic acid was detected with values of 2.5, 2.0 and 2.3 μg/g for Öelands hvede, Indigo and Konini, respectively. This was in agreement with those examined by Zhang et al., (2012) (2 to 8 μg/g) and Whent et al., (2012) (1.88 to 1.91 μg/g). Upon mixing flour formulation the content of free ferulic acid significantly ($p<0.05$) increased, up to 5-fold of the initial level. During fermentation, free ferulic acid kept increasing, meanwhile, some other free phenolic acid such as *p*-hydroxybenzoic acid, vanillic acid and *p*-coumaric acid were detected. This phenomenon confirmed the hypothesis that mixing and fermenting facilitated the release of bound phenolic compounds into free forms. *p*-Hydroxybenzoic, vanillic, *p*-coumaric and ferulic acid were all detected in the bread of three wheat varieties, indicating the remarkable effect of baking. Angioloni and Collar (2011) also indicated that some phenolic acids, such as protocatechuic, sinapic, syringic and ferulic acid were detected after bread-making but not in the raw flour. Free ferulic acid increased throughout the process of mixing, proofing and baking. This finding agreed with the study conducted by Hansen et al., (2002), who examined free ferulic acid in rye wholemeal. In bread samples, ferulic acid was predominant followed by *p*-hydroxybenzoic, vanillic and *p*-coumaric acid making up 45% to 51%, 26% to 37%, 11% to 18% and 4% to 5% of total phenolic acid content, respectively. Higher contents of ferulic and *p*-hydroxybenzoic acids were found in bread crumb than crust. This suggested that some free phenolic acids were thermally labile (high intense heat in bread crust). With respect to vanillic and *p*-coumaric acid, the levels were too low to make any conclusions.

Table 2.3 Composition of soluble-free phenolic acids in normal and purple wheat grains at different stages of bread-making process

Steps	Sample name	*p*-Hydroxybenzoic acid	Vanillic acid	*p*-Coumaric acid	Ferulic acid	Total phenolic acid
Flour	Öelands hvede	nd	nd	nd	2.50 ± 0.20 ^{J}	2.50 ± 0.20
	Indigo	nd	nd	nd	2.02 ± 0.07 ^{J}	2.02 ± 0.07
	Konini	nd	nd	nd	2.25 ± 0.01 ^{J}	2.25 ± 0.01
Mixing	Öelands hvede	nd	nd	nd	10.01 ± 1.23 ^{hi}	10.01 ± 1.23
	Indigo	nd	nd	nd	9.04 ± 0.24 ^{i}	9.04 ± 0.24
	Konini	nd	nd	nd	10.58 ± 0.69 ^{ghi}	10.58 ± 0.69
30 min Fermenting	Öelands hvede	nd	2.44 ± 0.53 ^{d}	1.31 ± 0.17 ^{ab}	11.25 ± 0.67 ^{egh}	15.01 ± 1.37
	Indigo	nd	nd	nd	10.57 ± 1.55 ^{ghi}	10.57 ± 1.55
	Konini	nd	nd	nd	12.55 ± 1.13 ^{ef}	12.55 ± 1.13
65 min Fermenting	Öelands hvede	4.19 ± 0.71 ^{ef}	3.49 ± 0.09 ^{c}	nd	13.34 ± 1.68 ^{cde}	21.03 ± 2.49
	Indigo	nd	nd	nd	13.25 ± 2.06 ^{de}	13.25 ± 2.06
	Konini	2.66 ± 0.37 ^{f}	nd	nd	15.01 ± 0.69 ^{sbcd}	17.67 ± 0.44
Bread Loaf	Öelands hvede	11.07 ± 2.42 ^{cd}	4.37 ± 0.29 ^{dc}	1.51 ± 0.07 ^{a}	13.53 ± 1.49 ^{cde}	30.47 ± 4.27
	Indigo	7.39 ± 2.41 ^{de}	5.20 ± 0.83 ^{ab}	1.26 ± 0.06 ^{b}	14.40 ± 0.42 ^{bcd}	28.25 ± 3.66
	Konini	11.87 ± 1.93 ^{bc}	3.44 ± 0.32 ^{c}	1.15 ± 0.01 ^{b}	15.53 ± 0.98 ^{ab}	32.00 ± 2.53
Bread Crust	Öelands hvede	nd	3.73 ± 0.27 ^{c}	nd	12.41 ± 0.90 ^{ef}	16.15 ± 1.17
	Indigo	nd	5.19 ± 0.04 ^{ab}	nd	12.01 ± 1.01 ^{efg}	17.21 ± 1.05
	Konini	nd	6.00 ± 0.28 ^{a}	0.70 ± 0.02 ^{c}	15.11 ± 0.26 ^{abc}	21.82 ± 0.55
Bread Crumb	Öelands hvede	16.29 ± 1.68 ^{a}	nd	nd	14.76 ± 0.74 ^{abcd}	31.05 ± 1.82
	Indigo	8.64 ± 1.87 ^{cd}	nd	nd	14.65 ± 0.03 ^{abcd}	23.29 ± 2.01
	Konini	15.24 ± 1.37 ^{ab}	nd	nd	16.44 ± 0.57 ^{a}	31.68 ± 1.54

Content of phenolic acid was expressed as ug/g of dry weight.

[a] Values in each column with different letters are significantly different ($p<0.05$).

Total phenolic acid was calculated as the sum of each row.

Table 2.4 summarizes the content of detected monomeric phenolic acids in insoluble fraction during the production of bread. Data on phenolic composition at each stage of bread-making in purple wheat were not previously available in the literature. Ferulic acid accounted for 77% to 82%, 78% to 83%, 77% to 83% of total bound phenolic acid in Öelands hvede, Indigo and Konini, respectively throughout the process of bread-making. This result was in accordance with previous studies on the phenolic profile of whole wheat (Okarter et al., 2010; Liu et al., 2010; Zhang et al., 2012). Moderate levels of phenolic acids were found as sinapic and *p*-coumaric acid, making up approximately 2.4% to 3.9% and 6.3% to 10.5% of total phenolic acids. The least abundant phenolic acids were protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic and syringic acid, having content lower than 20 μg/g.

In terms of bread-making process, mixing increased the content of seven phenolic acids, except for caffeic acid. The first 30 min fermenting step led to a significant ($p<0.05$) decrease in phenolic acids (only vanillic acid decreased slightly, $p>0.05$). Total phenolic acid decreased by 26% to 27% compared to the dough after mixing. The decrease was mainly attributed to the loss of ferulic acid. This phenomenon provided evidence for the release of bound phenolic acids into their free forms. However, when the dough was fermented for 65 min, the content of total phenolic acid conversely increased to almost the same level as the mixing dough. The prolonged fermentation step helped liberate the bond between phenolic acids and insoluble large compounds, such as dietary fibre, thus making it easier to extract the “freed” phenolics. Baking significantly ($p<0.05$) increased the content of phenolic acids, increasing up to 15% of total phenolic acid compared to the raw flour. Yu et al. (2013) also observed a significant increase of bound ferulic acid after baking.

With regards to individual phenolic acids, the content of protocatechuic and *p*-hydroxybenzoic acid became lower after baking. In bread crust, the content of protocatechuic acid was outside the detection limit, while *p*-hydroxybenzoic acid significantly ($p<0.05$) decreased. Higher levels of these two acids were found in bread crumb because of their indirect exposure to heat. This suggested that they are heat sensitive during thermal process. Similar conclusions could be made to vanillic and syringic acid, whose contents increased during baking but were detected in lower levels in bread crust. Most likely, these types of phenolic acids were still released through heating but were labile to intense heat. Therefore, at moderate level of heat, the content tended to slightly increase. However, when high heat was applied, they would degrade to their derivatives. Caffeic, *p*-coumaric, ferulic and sinapic acid, increased during baking and the contents were even higher in bread crust than crumb. This result was consistent with the finding of Moore et al., (2009) who reported that higher levels of insoluble bound ferulic acid were better recovered in baked pizza crust. The different behaviors of individual phenolic acids might be ascribed to their difference in chemical nature, sensitivity towards heat and ease of liberation from plant cell wall.

With respect to total phenolic acid content, the upper crust fraction contained the highest quantities of phenolic acids among other fractions (bread loaf and crumb). By comparison with free phenolic acid, the content of bound phenolic acid accounted for 97 to 99% of total phenolic acid content. This finding was in agreement with Zhang et al. (2012), who investigated 37 Chinese wither wheat cultivars, concluding that free phenolic acids made up of 2.5% of total phenolic content.

Table 2.4 Composition of insoluble-bound phenolic acids in normal and purple wheat grains at different stages of bread-making process

Steps	Sample name	Protocatechuic acid	*p*-Hydroxybenzoic acid	Vanillic acid	Caffeic acid	Syringic acid	*p*-Coumaric acid	Ferulic acid	Sinapic acid	Total phenolic acid
Flour	Öelands hvede	nd	22.73 ± 1.29 ^{b}	9.29 ± 0.54 ^{f}	4.54 ± 0.18 ^{k}	9.03 ± 0.58 ^{b}	26.13 ± 1.19 ^{a}	532.15 ± 30.00 ^{defgh}	69.36 ± 2.69 ^{cd}	673.25 ± 36.47
	Indigo	nd	25.69 ± 1.17 ^{a}	24.82 ± 0.01 ^{a}	6.53 ± 0.15 ^{ef}	9.95 ± 0.23 ^{a}	19.14 ± 1.50 ^{def}	447.02 ± 24.14 ^{kl}	35.97 ± 2.80 ^{k}	569.12 ± 29.99
	Konini	nd	24.60 ± 1.24 ^{a}	19.20 ± 0.97 ^{b}	7.10 ± 0.30 ^{bc}	9.05 ± 0.09 ^{b}	21.64 ± 0.68 ^{c}	466.66 ± 0.28 ^{jk}	52.48 ± 2.24 ^{hij}	600.74 ± 5.81
Mixing	Öelands hvede	20.67 ± 1.00 ^{a}	10.63 ± 0.73 ^{fg}	7.44 ± 0.49 ^{h}	4.85 ± 0.02 ^{jk}	9.46 ± 0.57 ^{ab}	26.50 ± 0.39 ^{a}	573.64 ± 11.84 ^{bcd}	72.37 ± 0.88 ^{bc}	725.56 ± 15.92
	Indigo	19.37 ± 0.60 ^{ab}	24.48 ± 1.68 ^{a}	9.48 ± 0.32 ^{f}	4.52 ± 0.00 ^{k}	5.97 ± 0.17 ^{f}	19.63 ± 1.25 ^{de}	482.89 ± 9.02 ^{ijk}	51.74 ± 2.88 ^{ij}	618.07 ± 15.94
	Konini	14.37 ± 0.84 ^{e}	20.59 ± 1.19 ^{c}	13.58 ± 0.18 ^{de}	5.70 ± 0.28 ^{g}	7.24 ± 0.36 ^{de}	22.57 ± 1.16 ^{bc}	514.29 ± 4.29 ^{hi}	60.38 ± 0.28 ^{ef}	658.73 ± 8.57
30 min	Öelands hvede	15.42 ± 0.43 ^{d}	7.83 ± 0.39 ^{h}	6.17 ± 0.17 ^{h}	nd	nd	21.02 ± 0.96 ^{cd}	411.57 ± 13.46 ^{lm}	64.66 ± 2.88 ^{de}	526.66 ± 18.23
	Indigo	15.46 ± 0.51 ^{d}	11.63 ± 0.31 ^{f}	8.68 ± 0.73 ^{fg}	nd	nd	14.56 ± 0.54 ^{ij}	363.99 ± 18.02 ^{n}	40.82 ± 3.24 ^{k}	455.14 ± 23.35
	Konini	11.13 ± 0.68 ^{f}	13.79 ± 0.42 ^{e}	12.76 ± 0.51 ^{e}	nd	nd	13.57 ± 0.03 ^{j}	386.39 ± 11.13 ^{mn}	49.10 ± 2.84 ^{j}	486.74 ± 15.60
65 min	Öelands hvede	17.56 ± 0.04 ^{c}	11.70 ± 0.61 ^{f}	12.86 ± 0.92 ^{e}	5.20 ± 0.08 ^{ij}	9.40 ± 0.46 ^{ab}	26.57 ± 0.56 ^{a}	526.64 ± 31.76 ^{efghi}	71.83 ±1.05 ^{bc}	681.75 ± 35.47
	Indigo	19.97 ± 0.23 ^{ab}	12.19 ± 0.24 ^{f}	19.18 ± 0.69 ^{b}	6.21 ± 0.28 ^{f}	6.83 ± 0.32 ^{e}	16.90 ± 0.25 ^{gh}	488.22 ± 2.63 ^{hijk}	54.86 ± 0.48 ^{ghi}	624.36 ± 5.12
	Konini	14.99 ± 0.22 ^{de}	15.77 ± 0.91 ^{d}	18.17 ± 0.98 ^{b}	6.86 ± 0.05 ^{cde}	7.82 ± 0.27 ^{cd}	18.61 ± 0.19 ^{efg}	523.25 ± 7.22 ^{fghi}	60.34 ± 2.47 ^{ef}	665.81 ± 12.31
Load	Öelands hvede	3.75 ± 0.12 ^{ghi}	10.94 ± 0.27 ^{fg}	14.68 ± 0.30 ^{cd}	5.30 ± 0.22 ^{hi}	9.63 ± 0.16 ^{ab}	26.64 ± 0.30 ^{a}	586.92 ± 30.32 ^{bc}	75.51 ± 2.09 ^{b}	733.38 ± 33.79
	Indigo	4.12 ± 0.08 ^{gh}	9.99 ± 0.32 ^{g}	19.32 ± 0.59 ^{b}	6.62 ± 0.16 ^{de}	6.97 ± 0.27 ^{e}	17.31 ± 0.33 ^{fgh}	517.14 ± 1.40 ^{hi}	56.12 ± 1.48 ^{fghi}	637.57 ± 5.16
	Konini	3.10 ± 0.08 ^{i}	5.29 ± 0.15 ^{ij}	18.65 ± 0.62 ^{b}	7.33 ± 0.26 ^{ab}	8.12 ± 0.09 ^{c}	19.39 ± 1.47 ^{de}	564.64 ± 37.89 ^{cdef}	65.31 ± 1.81 ^{de}	691.84 ± 43.37
Crust	Öelands hvede	nd	4.80 ± 0.21 ^{ij}	15.45 ± 0.21 ^{c}	5.66 ± 0.19 ^{gh}	nd	27.87 ± 0.79 ^{a}	631.54 ± 42.35 ^{a}	80.92 ± 3.40 ^{a}	766.23 ± 47.15
	Indigo	nd	4.44 ± 0.10 ^{j}	19.49 ± 0.66 ^{b}	7.17 ± 0.24 ^{bc}	nd	19.30 ± 1.07 ^{de}	551.35 ± 6.76 ^{cdefg}	57.45 ± 4.15 ^{fgh}	659.20 ± 12.98
	Konini	nd	4.25 ± 0.27 ^{j}	18.77 ± 0.63 ^{b}	7.63 ± 0.04 ^{a}	nd	20.70 ± 4.12 ^{cd}	616.95 ± 21.59 ^{ab}	71.57 ± 3.64 ^{bc}	739.88 ± 26.59
Crumb	Öelands hvede	3.95 ± 0.14 ^{ghi}	6.17 ± 0.59 ^{i}	14.51 ± 0.11 ^{cd}	5.19 ± 0.05 ^{ij}	9.80 ± 0.16 ^{a}	23.93 ± 1.64 ^{b}	569.32 ± 26.73 ^{cde}	70.96 ± 4.66 ^{bc}	703.82 ± 34.08
	Indigo	4.33 ± 0.17 ^{g}	5.62 ± 0.21 ^{ij}	18.33 ± 1.00 ^{b}	6.56 ± 0.15 ^{ef}	6.97 ± 0.38 ^{e}	17.10 ± 0.63 ^{gh}	498.80 ± 7.71 ^{hij}	51.14 ± 0.89 ^{ij}	608.85 ± 11.08
	Konini	3.25 ± 0.13 ^{hi}	4.32 ± 0.27 ^{j}	18.65 ± 1.21 ^{b}	6.96 ± 1.64 ^{bcd}	8.18 ± 0.13 ^{c}	16.05 ± 0.98 ^{hi}	554.64 ± 24.93 ^{cdefg}	58.22 ± 0.79 ^{fg}	670.28 ± 28.61

Content of phenolic acid was expressed as μg/g of dry weight.

[a] Values in each column with different letters are significantly different ($p<0.05$).

Total phenolic acid was calculated as the sum of each row.

2.4.5. Total Anthocyanin Content

Spectra of absorbance at different wavelengths were identified for anthocyanin extract. As displayed in **Figure 2.4**, a wavelength range of 520 to 535 nm was detected to exert the highest absorbance for anthocyanins in Indigo and Konini varieties. No obvious peak was observed for Öelands hvede flour because of the low anthocyanin content. Giusti and Wrolstad (2005) summarized the $\lambda_{(vis\text{-}max)}$ for cyanidin-3-glucoside using different solvents, indicating the $\lambda_{(vis\text{-}max)}$ of 510 nm for aqueous buffer, 512 nm for 10% ethanol, 520 nm for 0.1N HCl and 530 nm for 1% HCl in methanol. Therefore, wavelengths of 520 and 535 were used for detecting anthocyanins in acidified methanol extracts.

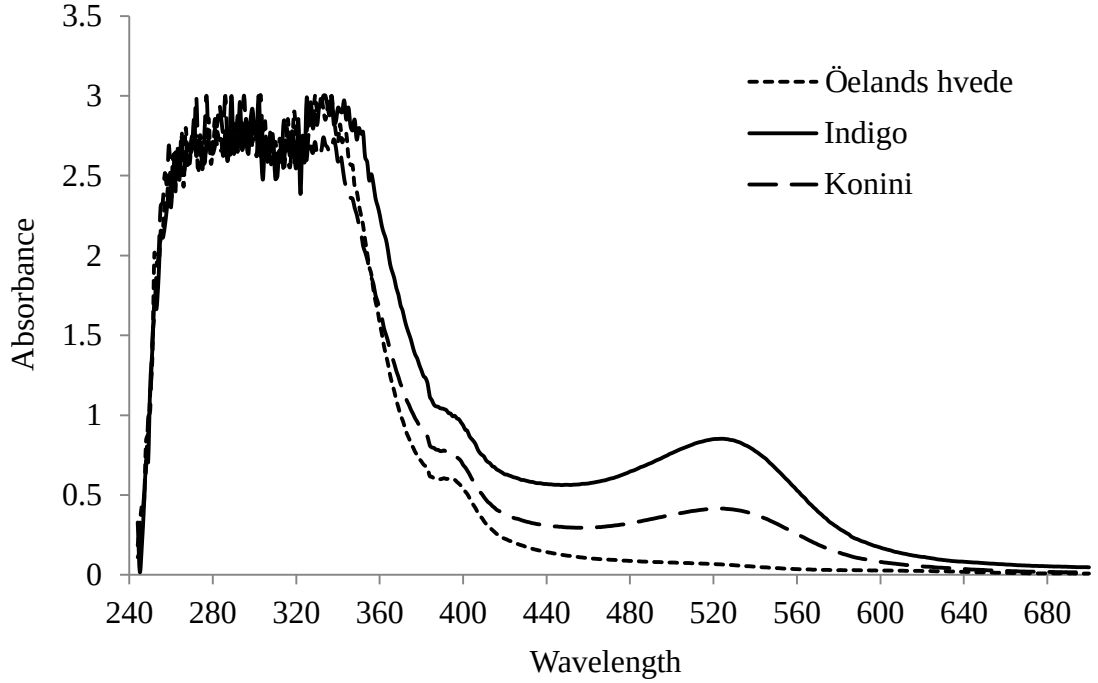


Figure 2.4 UV/Visible spectra of raw wheat flour at different wavelengths

Table 2.5 Total anthocyanin content in normal and purple wheat grains at different stages of bread-making process

	Öelands hvede	Indigo	Konini
Flour*	1.45 ± 0.06^{k}	10.84 ± 0.27^{a}	5.61 ± 0.12^{e}
Mixing*	1.06 ± 0.05^{l}	8.50 ± 0.14^{d}	4.38 ± 0.05^{h}
30min Fermenting*	1.35 ± 0.03^{k}	8.95 ± 0.09^{c}	5.05 ± 0.14^{fg}
65min Fermenting*	1.39 ± 0.03^{l}	9.47 ± 0.31^{b}	5.20 ± 0.04^{f}
Bread Loaf*	1.02 ± 0.03^{l}	4.85 ± 0.03^{g}	2.60 ± 0.03^{i}
Bread Crust*	0.86 ± 0.06^{l}	4.39 ± 0.14^{h}	2.33 ± 0.05^{j}
Bread Crumb*	0.55 ± 0.02^{m}	5.47 ± 0.03^{e}	2.77 ± 0.06^{i}

*Results were expressed as mg cyanidin-3-glucoside equivalents / 100g.

[a] Values with different letters are significantly different ($p<0.05$).

Indigo contained significantly ($p<0.05$) higher anthocyanin content throughout the whole process of bread-making, ranging from 4.39 to 10.84 mg C3GE/100g (**Table 2.5**). The lowest TAC was obtained from bread crust and the highest was from raw flour. The TAC values for Konini varied from 2.33 to 5.61 mg C3GE/100g with lowest in bread crust and highest in raw flour. The non-colored wheat, Öelands hvede contained the lowest TAC, which was approximately 8 and 4 times lower than the raw flours of Indigo and Konini, respectively.

With respect to bread-making process, mixing significantly ($p<0.05$) reduced TAC values. This was probably due to the dilution of anthocyanin content with other bread ingredients. Fermentation induced the release of anthocyanins and TAC gradually increased during fermentation. This effect was significantly ($p<0.05$) seen at both 30 min and 65 min fermenting steps. The TAC value increased up to 19% compared to the dough after mixing. Baking had a significant ($p<0.05$) influence on anthocyanins, leading to 54% to 55% decrease of TAC levels in Konini and Indigo varieties, respectively. This result was in

agreement with the study of Jing et al. (2007), who stated that anthocyanins were relatively unstable and often underwent degradation during processing. Abdel-Aal and Hucl (2003) investigated the stability of anthocyanins at different temperatures. They found that when temperature increased from 65 to 95 ^{o}C, the degradation also increased. Li et al. (2007) reported a complete destruction of anthocyanins from purple wheat bran when heating processing (177 ^{o}C for 7-12 min) was used for muffin production. In the present study, the thermally labile property of anthocyanins was further confirmed by the TAC of bread fractions. Bread crust, which was exposed to the intense heat, contained the lowest TAC value. Bread crumb contained a relatively higher TAC value because the inner-side of bread possessed a less intense heat. In terms of Öelands hvede variety, TAC was relatively stable throughout the whole process of bread-making. This indicated the minor level of anthocyanins in Öelands hvede.

2.4.6. Total Anthocyanin Content using pH Differential Method

Table 2.6 Total anthocyanin content using pH differential method

	Öelands hvede	Indigo	Konini
Flour*	nd	10.41 ± 0.39^{a}	5.31 ± 0.02^{d}
Mixing*	nd	8.09 ± 0.37^{c}	4.22 ± 0.07^{f}
30min Fermenting*	nd	8.60 ± 0.47^{bc}	4.65 ± 0.25^{ef}
65min Fermenting*	nd	8.97 ± 0.26^{b}	4.80 ± 0.08^{de}
Bread Loaf*	nd	4.64 ± 0.24^{ef}	2.15 ± 0.04^{g}
Bread Crust*	nd	4.16 ± 0.21^{f}	1.99 ± 0.07^{g}
Bread Crumb*	nd	4.81 ± 0.26^{de}	2.18 ± 0.08^{g}

*Results were expressed as mg cyanidin-3-glucoside equivalents / 100g.

[a] Values with different letters are significantly different ($p<0.05$).

Table 2.6 summarized the total anthocyanin content using pH differential method.

Similar results were obtained using this method. TAC significantly ($p<0.05$) decreased after mixing and gradually increased during fermenting. Baking destroyed anthocyanins and the exposure to intense heat led to the lowest TAC in bread crust. Compared to the result using direct measurement method, pH differential method detected lower TAC in Indigo and Konini extracts and detected no anthocyanins in Öelands hvede. This method is more accurate for determining TAC, since two aspects have been improved by pH differential method: 1) suppressed the interference of degraded anthocyanins and its derivatives; 2) omitted the hazes or sediments.

2.4.7. Antioxidant Activity of Anthocyanin Extracts

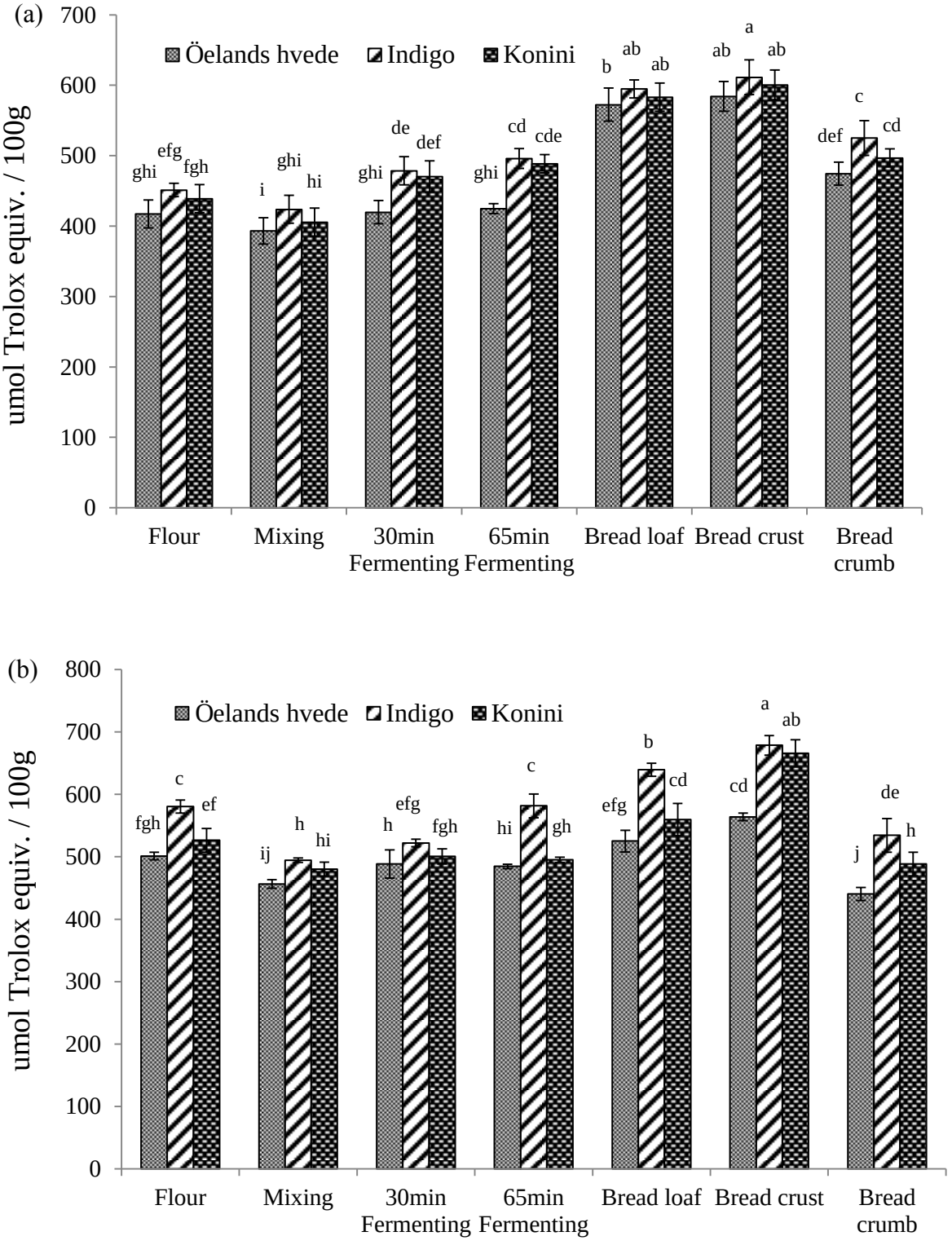


Figure 2.5 DPPH radical scavenging capacity (a) and ABTS radical cation decolorization activity (b) of anthocyanin extracts. Columns labeled with different letters are significantly different ($p<0.05$).

Anthocyanin extracts during the production of bread were examined for their AOA against DPPH radical and ABTS radical cation (**Figure 2.5**). The DPPH values of purple wheat varieties were higher than that of the normal wheat (**Figure 2.5a**). The significant ($p<0.05$) difference was detected in fractions from 30 min and 65 min fermenting steps. The high AOA of purple wheat was most likely contributed to the presence of anthocyanins. Consistent to TAC, DPPH values decreased after mixing and gradually recovered during fermentation. Significant ($p<0.05$) increase of 37, 32 and 33% for Öelands hvede, Indigo and Konini, respectively in AOA was observed in bread. Bread crusts exhibited the highest values. This could be explained by the extracting method, which not only targeted anthocyanins, but also extracted other soluble antioxidants such as phenolic acids and MRPs.

The effect of anthocyanins on AOA was better revealed by $ABTS^{\bullet+}$ decolorization activity assay (**Figure 2.5b**). Significant ($p<0.05$) difference was detected between the purple and common wheat varieties. Indigo exhibited the highest ABTS values. The changes in ABTS levels during bread production were in accordance with DPPH assay.

2.4.8. Analysis of Anthocyanin Composition

To examine the presence of anthocyanins, HPLC chromatogram with MS and MS/MS was recorded at wavelength of 520 nm. Anthocyanin composition detected by HPLC-QTOF-MS/MS is shown in **Figure 2.6**. Only one main peak was recognized as anthocyanin using MS/MS, namely cyanidin-3-glucoside (RT = 22.48 min). Two ions were evident for the identification including *m/z* 499 ($[M+H]^+$) being the molecular weight of anthocyanin, and fragment ion *m/z* 287 arising from a loss of glucose residue *m/z* 162. This was in agreement

with the study of Hosseinian et al. (2008). Liu et al. (2010) also detected cyanidin-3-glucoside in Indigo and Konini wheat without the presence of other anthocyanins.

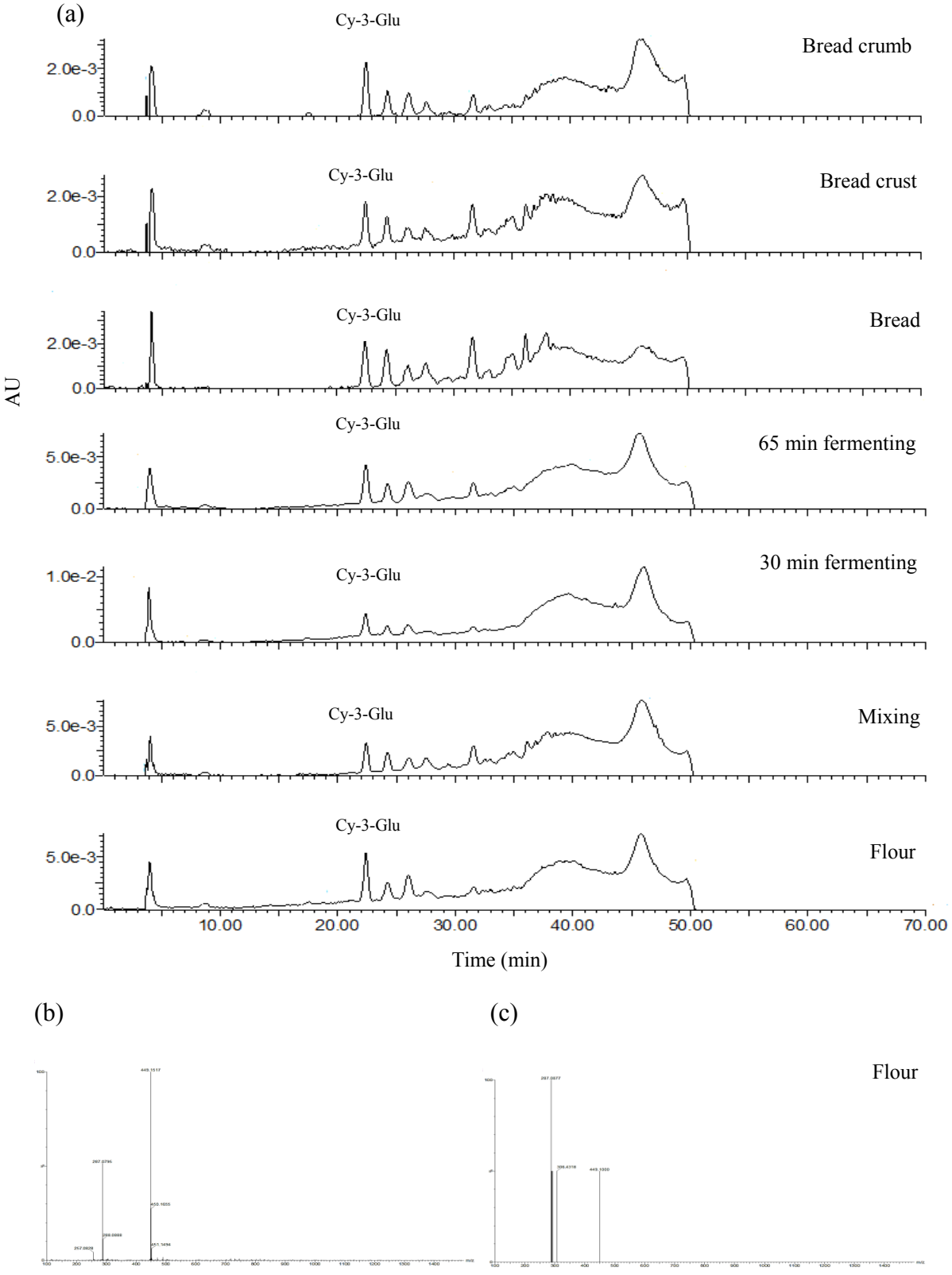


Figure 2.6 LC chromatogram (a) of anthocyanin at different stages of bread-making as well as LC-MS (b) and LC-MS/MS of cyanidin-3-glucoside (cy-3-glu).

2.4. Conclusions

In summary, the changes of FPC, BPC, TAC as well as their AOA during the production of bread were first systematically investigated. Individual phenolic acids were also identified and quantified in fractions from each stage of bread-making. Mixing, fermenting and baking significantly increased FPC and BPC. Bread crust contained the highest FPC, while bread crumb contained the highest BPC. AOA were correlated to their respective phenolic content. Different phenolic acids behaved differently in the bread-making process, which might be ascribed to their difference in chemical natures, sensitivities towards heat and ease of liberation from plant cell walls. TAC was significantly reduced through mixing and baking, but fermentation elevated the level of TAC. Anthocyanin extract of purple wheat exerted higher AOA than the common wheat, indicating the potential of purple wheat to be used as a functional ingredient in baking.

CHAPTER III. Identification and Comparison of Antioxidant Properties of Phenolic Compounds in Purple Wheat Bread after Digestion using *In-Vitro* Human Gastrointestinal Tract Model

3.1. Abstract

Increasing evidence has shown the health benefits of phenolic compounds in cereal grains. However, their beneficial effects in human bodies depend on their metabolic fate in the human gut and ultimately their bioavailability. The objectives of the present study were to investigate the antioxidant properties of bread extracts using simulated human gastrointestinal (GI) conditions and examine the effects of the digested phenolic compounds using cell-based models of cancer and normal human intestinal cells. The *in-vitro* digestion model was based on simulation of human GI tract with various enzymes functioning at their optimum pHs. Three bread wheat cultivars were investigated, i.e. Oelandshvede (normal), Indigo (purple) and Konini (purple). Digested bread extracts were evaluated for total phenolic content (TPC), DPPH• scavenging capacity activity, ABTS•+ decolorization capacity and HPLC analysis. Three human gut cell lines were selected to evaluate their intracellular effects (Caco-2 and HT-29, cancer cell lines and FHs 74 Int, non-transformed cell line). Cytotoxicity, cytoprotective activity against AAPH and H_2O_2-induced oxidation and dichlorofluoresin diacetate (DCF) assays were employed. Digested sample extracts of phenolic compounds from the purple wheats, Indigo and Konini, showed significantly ($p<0.05$) higher TPC (166 & 126 mg ferulic acid equiv/100g) than common wheat bread (120 mg ferulic acid equiv/100g). HPLC analysis detected protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric and ferulic and sinapic acids with total values ranging from 22 to 30 ug/g. ABTS and DPPH values ranging from 419 to 461 and 125 to 207 umol TE/g

respectively, correlated to TPC. Purple wheat bread had higher cellular antioxidant activity (CAA) than common wheat bread. Cancer cell lines exhibited maximum cell viability at concentration of 100 mg/ml for cytoprotective activity against both AAPH and H_2O_2, while normal cell lines showed an optimum viability at concentration of 20 mg/ml. The DCF assay demonstrated that 5 mg/mL extracts for HT-29 and Caco-2 cell lines and 500 mg/ml extracts for FHs cells exhibited antioxidant properties. Purple wheat bread demonstrated higher *in-vitro* and cellular antioxidant properties than common wheat after simulated human GI digestion, which indicates its potential to be used as a functional ingredient for cereal grain-based baked foods.

3.2. Introduction

Bread, as a major product from wheat, is an important component in the daily diet of many countries (Gawlik-Dziki et al., 2009). However, due to the complexity of bread-making process, the amount of phenolic compounds delivered by bread may differ quantitatively and qualitatively from what has been compiled in the food database.

The beneficial effects of wheat phenolic compounds in biological environment are also determined by bioavailability and metabolic fate. In the literature, the functional properties of phenolics were mainly reported in organic solvent extracts, therefore lacking representation of conditions of the human gut. The complexity of *in-vivo* biological system makes it hard to standardize the digestive conditions, thus creating less consistent comparisons from lab-to-lab (Coles et al., 2005). Nowadays, models under simulated GI conditions have been regarded as simple, inexpensive and possible reproducible systems for

investigating the structural changes, release and digestibility of food components (Oomen et al., 2002). This technique utilizes the major enzymes existent in oral, gastric and intestinal pathways, and is basically constructed upon starch digestion by α-amylase, lipid digestion by lipase and protein digestion by pepsin or trypsin (Hur et al., 2011). Simulated GI tract model is considered to be closer to the human gut environment and data obtained from digested edible goods are more practical for nutritional purpose.

To properly assess the health potential of phenolic extracts, cell cultures are widely utilized to predict their possible effects in living organisms. However, in most studies, cell culture models combined as part of *in-vitro* GI digestion are usually used to predict the absorption of bioactive compounds, such as iron uptake or for pharmaceutical preparation purpose (Hur et al., 2011). There is need for further investigations of antioxidant activity and cytoprotective properties of phenolic compounds released from a ready-to-eat food product during *in-vitro* digestion conditions.

Our current study aimed to: (1) characterize the phenolic profiles in the extracts of *in-vitro* digested bread samples derived from purple wheat varieties; (2) investigate the antioxidant properties and cytoprotectivity using both chemical and cell based assays.

3.3. Materials and Methods

3.3.1. Chemicals

(1) Extraction

Mucin, alpha-amylase, pepsin, pancreatin and bile salts were purchased from Sigma-Aldrich

Chemical Co. (St. Louis, MO, USA). HPLC grade ethyl acetate was purchased from Fisher Scientific Co. (Ottawa, ON).

(2) Chemical assays and HPLC analysis

Materials utilized for chemical assays and HPLC analysis are listed in section 2.3.1.

(3) Cell culture assay

Cell growth reagents, including fetal bovine serum (FBS), penicillin/streptomycin (P/S), L-glutamine (L-gln), sodium pyruvate (Na/Pyr), human transferrin (hTF), epidermal growth factor (EGF) and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Trypsin-EDTA used for cell dissociation and Dulbecco's modified eagle medium with D-glucose content (DMEM) used for growing cancer cell lines were obtained from Invitrogen Canada Inc. (Burlington, ON, Canada). Hybri-care medium (ATCC®, 46-X^{TM}) used for growing FHs 74 Int cell line and MTT cell proliferation kit (ATCC® 30-1010k) used for cell viability assay was from ATCC (American Type Culture Collection; Manassas, VA, USA). 2$^{'}$,7$^{'}$ -Dichlorofluorescin diacetate (DCFH-DA) was purchased from Invitrogen Canada, Inc. (Burlington, ON, Canada).

3.3.2. Sample Description

The wheat varieties are described in chapter 2.3.2.

3.3.3. Bread-Making Procedure and Sample Preparation

Bread was made and freeze-dried following the method described in sections 2.3.4 and 2.3.5.

3.3.4. In-Vitro Gastrointestinally Digested Extraction

3.3.4.1. Simulated gastrointestinal fluid

The GI digestion was performed according to Swieca et al. (2013) with some modifications. Three solutions were prepared to simulate the digestion in mouth, stomach and intestine. The formulas were described as followings:

Saliva fluid was prepared by dissolving 1.19 g Na_2HPO_4, 0.095 g KH_2PO_4, 4 g NaCl and 50 g mucin in 500 mL of distilled water. The pH was adjusted to 6.75 and α-amylase was added to obtain a 200 U per mL of enzyme activity.

Gastric juice was prepared by adding 1 g NaCl, 1.6 g pepsin, and 3.5 mL HCl into 500 mL distilled water. The solution was adjusted to pH = 1.2.

Intestinal juice was prepared by dissolving 0.15 g pancreatin and 0.9 g bile extract in 105 mL 0.1 mol/L $NaHCO_3$.

3.3.4.2. In-vitro digestion

The *in-vitro* digestion was performed according to the following steps:

1) 5 g of ground bread sample was homogenized with 15 mL of simulated salivary fluid using a vortex for 1 min. Subsequently, the mixture was shaken for 10 min at 37 °C in a water bath at 150 rpm speed (VWR, Radnor, PA).
2) The pH of the mixture was adjusted to 1.2 using 6 N HCl followed by adding 15 mL of simulated gastric juice. The sample was then shaken for 60 min at 37 °C using the same water bath (VWR, Radnor, PA).
3) The sample was adjusted to pH 6 with 0.1 mol/L $NaHCO_3$, followed by addition of 15 mL of intestinal juice. The pH was then adjusted to pH 7 with 4 mol/L NaOH.

Subsequently, 5 mL of 120 mmol/L NaCl and 5 mL of 5 mmol/L KCl were added to the sample. Continuous shaking of the sample was performed in darkness for 120 min at 37 °C. Thereafter, the sample was transferred to an ice bath to terminate the enzyme reaction and then centrifuged at 11,963 g, 4 °C for 20 min (Sorvall RC 6+, Thermo Fisher Scientific, Ottawa, Ontario). The supernatant was collected and used for further extraction.

3.3.4.3. Extraction of phenolic compounds

The digested mixture was adjusted to a pH between 1.5 and 2.0 using 6 N HCl and then defatted twice with hexane (25 mL each time). The hydrophilic part was separated from hexane and subsequently extracted three times with ethyl acetate (25mL each time). Every time after mixing, ethyl acetate was separated from the aqueous layer by centrifuging at 11,963 x g and 4 °C (Sorvall RC 6+, Thermo Fisher Scientific, Ottawa, Ontario) for 5 min. The supernatant was collected and further dehydration was performed by adding 1 g of Na_2SO_4 before filtering with a filter paper (90 mmφ, Whatman^{TM} Cat No. 1004 125). The combined ethyl acetate extracts were evaporated and dried at 35 °C under vacuum using a rotary evaporator (Yamato RE-51, Cole-Parmer Instrument Company, IL, USA) with a water bath (Thermo-Lift, Fisher Scientific, NJ, USA). The residue was then redissolved in 2 mL of 50% methanol for the determination of total phenolic content, DPPH radical scavenging capacity, ABTS radical cation decolorization assay, oxygen radical absorbance capacity (ORAC) and HPLC analysis. For cell culture assays, the residue was redissolved in 5 mL of phosphate buffered saline (PBS). Extraction was done in duplicate.

3.3.5. Chemical Model Assays

3.3.5.1. Determination of phenolic content

Total phenolic contents were determined using the method described in section 2.3.9.

3.3.5.2. DPPH radical scavenging capacity activity assay

DPPH values were determined following the method explained in section 2.3.10.

3.3.5.3. ABTS radical cation decolorization assay

The ABTS assay described in section 2.3.11 was utilized.

3.3.5.4. Oxygen radical absorbance capacity assay (ORAC)

The antioxidant activity was determined using the ORAC assay described by Huang et al. (2002) with some modifications. The assay was performed in a 96-well flat bottom polystyrene microplate (Corning Incorporated, Corning, NY, USA). A Precision 2000 automated microplate pipetting system (Bio-Tek Instruments, Inc., Winooski, VT, USA) was used to add 150 uL fluoroscein with 25 uL extracts or trolox standard into each well. After shaking for 3 min, the plate was incubated at 37 °C for 15 min. Then, 25 uL AAPH was added to each well and the fluorescence intensity was measured automatically by a FLx800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) controlled by software KC4 3.0 with an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm every minute. A regression equation between the trolox concentration and the net area under the fluorescence decay curve was obtained. Therefore,

$$AUC = 0.5 + f1 / f0 + \ldots + fi / f0 + \ldots + f49 / f0 + 0.5 \times (f50 / f0)$$

Where, *f*0 = initial fluorescence reading at *0* min and *fi* = fluorescence reading at time *i* min.

The final results were expressed as Trolox equivalent and were determined based on the standard curve.

3.3.6. HPLC Analysis

HPLC analysis was carried out using the method described in section 2.3.15.

3.3.7. Cell Culture Assays

3.3.7.1. Cell lines and cell culture

Three human gut cell lines were selected for the evaluation of *in-vitro* digested bread extracts. Caco-2 and HT-29 cell lines were both derived from human caucasian colon adenocarcinoma, and FHs 74 Int cell line which originated from the small intestine of a healthy 3 to 4 month female baby. These cell lines were obtained from ATCC (American Type Culture Collection; Manassas, VA, USA). The cells used in the present study were between generations of 11 to 16 for Caco-2 cells, 37 to 42 for HT-29 cells and 1 to 6 for FHs 74 Int cells.

3.3.7.2. Growth of cell lines

The Caco-2 and HT-29 cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, cell culture medium with D-glucose content) supplemented with 10% fetal bovine serum (FBS, growth promoting and survival enhancing factors), 10 mg/mL penicillin/streptomycin (antibiotics against bacterial contaminations), 200 mM L-glutamine (energy source), 100 mM sodium pyruvate (source of energy), and 1 mg/mL human

transferrin (ion-delivery agent binds). To grow FHs 74 Int cell line, a Hybri-care medium (American Type Culture Collection (ATCC), 46-X^{TM}, Manassas, VA) was used as the cell culture medium with the essential supplement of 10% fetal bovtine serum, 1% penicillin/streptomycin and 10 μM epidermal growth factor (EGF, extra growth factor that increase the rate of cell proliferation). The cells were maintained at 37 ºC in a 5% CO_2 humidified incubator.

The cell culture medium was changed three times a week to maintain the optimum growth of cells. Trypsin with the addition of EDTA was used to detach the cells from the cell culture flask for sub-cultivation when the cells reached an 80% to 90% confluence. Cell culture medium was used to rinse out the trypsin solution and resuspend the cells during routine passage.

For cell culture assays, intestinal cell line suspension (100 μL) was seeded in a 96-well plate at a concentration of 1×10^2/mL for at least 72 h until each well was fully covered with cells.

3.3.7.3. Cytotoxicity assay

Various concentrations of *in-vitro* digested bread samples, AAPH and H_2O_2 were tested for their cytotoxicity using the MTT cell proliferation assay according to a method described by Hirawan et al. (2011) with some modifications. Four dilutions of sample treatment were made (500 mg/ mL, 100 mg/mL, 20 mg/mL, 5 mg/mL) with PBS (medium). Various concentrations of AAPH and H_2O_2 were made with PBS, including 0, 1, 3, 5, 10, 15, 20 25 mM and 0, 1, 3, 5, 10 mM for AAPH and H_2O_2, respectively. The medium in all wells of the 96-well plate were aspirated. Then 100 μL of each extract/oxidant was plated into the well and fresh PBS

was used as control. The plate was covered with aluminum foil. After incubation for 4 h at 37 ºC, MTT reagent (10 μL) was added to all the wells and left in the incubator for approximately 3 h until the intracellular punctate purple precipitate was visible under the microscope. Detergent reagent (100 μL) was added to all the wells. The covered plate was then left at room temperature for 15 min and the measurement was taken at 590 nm using an Opsys MR 96-well plate reader (Dynex Technologies, Chantilly, VA). Cytotoxicity level was calculated as: Cell viability (%) = (absorbance of treatments) / (absorbance of controls) × 100%. Analysis was done in six replicates.

3.3.7.4. Cytoprotective activity against AAPH-induced oxidation

The cytoprotectivities of digested bread extracts against AAPH-induced oxidation was determined according to a method described by Gliwa et al. (2011) with some modifications. Specifically, after aspirating the entire medium, the digested bread extracts (100 μL) were added to sample wells and 100 μL of PBS was added to the control and blank wells. After 2 h of incubation, 10 μL of 3 mM AAPH was added to all the wells except for negative control and blank wells. Cells were then incubated for another 4 h. MTT reagent (10 μL) was then added to all except the blank wells and the plate was incubated for approximately 3 h until the intracellular punctate purple precipitate was visible under the microscope. The plate was then aspirated gently and the detergent reagent was added to all except the blank wells. After standing overnight at room temperature, the absorbance was recorded at 560 nm (Opsys MR 96-well plate reader, DYNEX Technologies, Chantilly, VA, USA). The cytoprotective activity of digested bread extracts against AAPH-induced oxidation was expressed as cell

viability (%) = (absorbance of treatments) / (absorbance of controls) × 100%. Analysis was done in six replicates.

3.3.7.5. Cytoprotective activity against H_2O_2-induced oxidation

The cytroprotective activity against H_2O_2-induced oxidation assay was formed using the procedure described in 3.2.8.4, but 1 mM of H_2O_2 was used instead of 3 mM of AAPH. Analysis was done in six replicates.

3.3.7.6. Cellular antioxidant activity (CAA) assay using dichlorofluoresin diacetate as fluorescent probe

The digested bread samples were tested for their effects in cellular oxidative status using a CAA method with some modifications (Hirawan et al. 2011). The medium in the entire well was aspirated and 100 μL of each extract was added for sample well, while 100 μL of fresh medium was added for positive control, negative control and blank wells. The cells were treated and left in the incubator for 1 h. At 30 min of incubation, 100 μL of 10 μM DCFH-DA solution was added to all wells except for the blank. The final concentration of the DCFH-DA solution would be 5 mM. After 1 h of incubation, all 200 μL of treatment solutions were removed, and 100 μL of 500 μM AAPH dissolved in PBS was added to all the wells except for negative control and blank wells (100 μL of PBS was added instead). The plate was then immediately placed into a Fluoroskan Ascent FL 96-well plate reader (ThermoLabsystems, Franklin, MA). Temperature was set at 37 °C, emission wavelength at 527 nm, and excitation wavelength at 485 nm, and measurement was taken every 30 min for 2 h. Analysis was done in six replicates.

The CAA value was calculated by integrating the area under the curve of fluorescence versus time at each concentration of digested bread sample extracts as:

$$\text{CAA value} = [\ 1- (\ \int \text{SA} / \int \text{CA})] \times 100$$

where SA is the area under sample fluorescence versus time curve and CA is the area under positive control fluorescence versus time curve.

3.3.7.7. Cell culture for reactive oxygen species (ROS) imaging

Microscopic image of real time cells oxidizing HDCF-DA in the presence of AAPH was conducted. *In-vitro* digested bread samples were tested for their effect in cellular oxidative status using CAA with DCFH-DA method. Specifically, HT-29 cell line was grown, trypsinized, washed and plated onto 22 mm^2 sterile cover slide. Cells were left to grow to 90% confluence on Inoue chamber at 37 °C in a 5% CO_2 humidified incubator. The medium from all wells was aspirated. Then, 100 µL of 5 mg/mL sample extract and 100 µL of PBS (control) were added. The cells were treated and left for 30 min of incubation. DCFH-DA solution (100 µL of 10 µM) was then added to the chambers. After 1 h of incubation, all 200 µL of treatment solutions were removed and 100 µL 500 µM AAPH dissolved in PBS was added to all chambers. The cells were then viewed with fluorescent microscopy (Olympus IX-70) connected to the Xenon Arc Lamp (Sutter DG4), computer, camera (Hamamatsu C4880-80). The wavelengths were set at emission wavelength at 527 nm, and excitation wavelength and measurements were taken every 30 min for 2 h.

3.3.8. Statistical Analysis

Experimental data were expressed as mean ± standard deviation (SD) of duplicate

determinations. Two way analysis of variance (ANOVA) test with SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) was employed for main factors (concentrations, bread type, concentration*bread type). Statistical significance was determined through Least Significant Differences (LSD) at a level of $p<0.05$. Pearson correlation test was used to evaluate the correlation among variables at significant level of $p<0.05$.

3.4. Results and Discussion

3.4.1. Total Phenolic Content of Digested Bread Samples

As seen in **Table 3.1**, TPC of digested bread varied from 119, 126 to 166 mg FAE / 100g (92, 97 to 125 mg GAE / 100g) for Öelands hvede, Konini and Indigo, respectively. Among them, the purple wheat variety – Indigo exhibited a significantly ($p<0.05$) higher TPC than Konini and Öelands hvede. The control wheat - Öelands hvede contained the lowest value. The investigation of bread using conversional extracting method (**Table 2.2**) exhibited TPC of 344, 340 and 331 FAE / 100g for Öelands hvede, Konini and Indigo, respectively. Phenolic bioaccessibility could be obtained by comparing the TPC before and after digestion. The purple wheat, Indigo demonstrated the highest bioaccessibility, making up 50% of its total phenolic content. While, Konini and Öelands hvede had bioaccessibilities of 37% and 35%, respectively.

Angioloni and Collar (2011) examined the bread of commercial white wheat and buckwheat using *in-vitro* digestion model, reporting TPC of 68.5 and 80.8 mg GAE / 100g, respectively. Nevertheless, ten-fold higher TPC (1698 mg GAE / 100g) was reported by Swieca et al. (2013) for wheat bread after *in-vitro* GI digestion. Other studies also assessed

TPC of digested bread samples, for example, 0.62 mg GAE/mL of digested fluid (Gawlik-Dzikl et al., 2009; Hiller et al., 2011); however, the use of different units made it hard to compare with present study. The use of different types and quantities of enzymes in simulated GI tract model determined the variations in TPC. Yu et al. (2013) studied the TPC of whole wheat bread using acidified ethanol through conversional extraction; the values obtained ranged from 80 to 160 mg FAE / 100g. The simulated human GI system in the present study involved release of ester-linked or bound phenolics by various enzymes, leading to higher TPC.

Table 3.1 Total phenolic content and antioxidant activities of digested bread samples

	TPC (mg FA equiv. / 100g DW)	**TPC** (mg GA equiv. / 100g DW)	**DPPH** (umol Trolox equiv. / 100g DW)	**ABTS** (umol Trolox equiv. / 100g DW)	**ORAC** (umol Trolox equiv. / 100g DW)
Öelands hvede	119.95 ± 5.92^{b}	92.42 ± 4.11^{b}	125.13 ± 4.55^{c}	419.63 ± 21.43^{a}	4367.99 ± 311.44^{b}
Indigo	166.47 ± 6.58^{a}	124.68 ± 4.56^{a}	207.08 ± 11.36^{a}	461.31 ± 12.50^{a}	5040.53 ± 326.25^{a}
Konini	126.23 ± 4.28^{b}	96.77 ± 2.97^{b}	173.34 ± 9.09^{b}	438.57 ± 19.65^{a}	4712.02 ± 197.27^{ab}

[a] Values in each column with different letters are significantly different ($p<0.05$).

3.4.2. Antioxidant Activity of Digested Bread Samples

Results from DPPH, ABTS and ORAC assays are presented in **Table 3.1**. Among the three wheat varieties, Indigo exhibited significantly ($p<0.05$) higher DPPH value of 207, while Konini demonstrated 173 umol TE/100g. Öelands hvede had a value of 125 umol TE/100g, which was the significantly ($p<0.05$) lower than the purple wheat varieties. Due to the use of different units and examining procedures, no reference was found for DPPH value of digested bread extract. Whole wheat bread samples were assessed for free and bound fractions in the previous chapter (**Figure 2.1** and **2.2**) and the resultant total DPPH values

were 689, 631 and 652 μmol TE/100g for Öelands hvede, Indigo and Konini, respectively. The digested extract exerted lower AOA due to the incomplete extraction of phenolics using simulated GI fluids. The %AOA of digested samples, based on total DPPH levels, were 33% and 27% for Indigo and Konini, respectively. The digested extract of common wheat, Öelands hvede had a much lower %AOA, making up 18% of its total DPPH level.

ABTS assay presented a higher magnitude than DPPH assay, ranging from 419 to 461 μmol TE/100g. Indigo had the highest while Öelands hvede contained the lowest value; however, no significant difference ($p>0.05$) was found among the three wheat varieties. Similar to DPPH, higher ABTS values were reported in **Figure 2.1** and **2.2**. The total ABTS values (free and bound) varied from 1671, 1571 and 1603 μmol TE/100g for Öelands hvede, Indigo and Konini, respectively. Among the three wheat varieties, Indigo had the lowest AOA before but highest after digestion, whereas the common wheat exhibited a reversal of results. This indicated that purple wheat might pose better protection than common wheat against free radical induced oxidation in the human GI tract.

ORAC values for digested bread samples ranged from 4368 to 5041 μmol TE / 100g. Still, Indigo had the highest while Öelands hvede contained the lowest ORAC values. Yu et al. (2013) examined the ORAC values for whole wheat bread, revealing a range of 5189 to 6465 μmol TE / 100g. A significantly lower value (2376 ± 164 umol TE/100g) was reported by Michalska et al. (2008) for rye bread. Moore and Yu (2008) also reported lower ORAC value (2000 μmoles TE / 100 g) for whole wheat bread. The variations were most likely due to the use of different raw materials, bread-making procedures and extracting methods. Results from both TPC and AOA showed that purple wheat has potential to be used as a

functional food ingredient compared to common wheat.

3.4.3. Analysis for Phenolic Acids Composition using HPLC

Figure 3.1 showed the chromatograms of digested bread extracts. The detected monomeric phenolic acids were labeled and numbered according to the retention time. Except for gallic acid, other eight phenolic acids were detected in the digested bread samples.

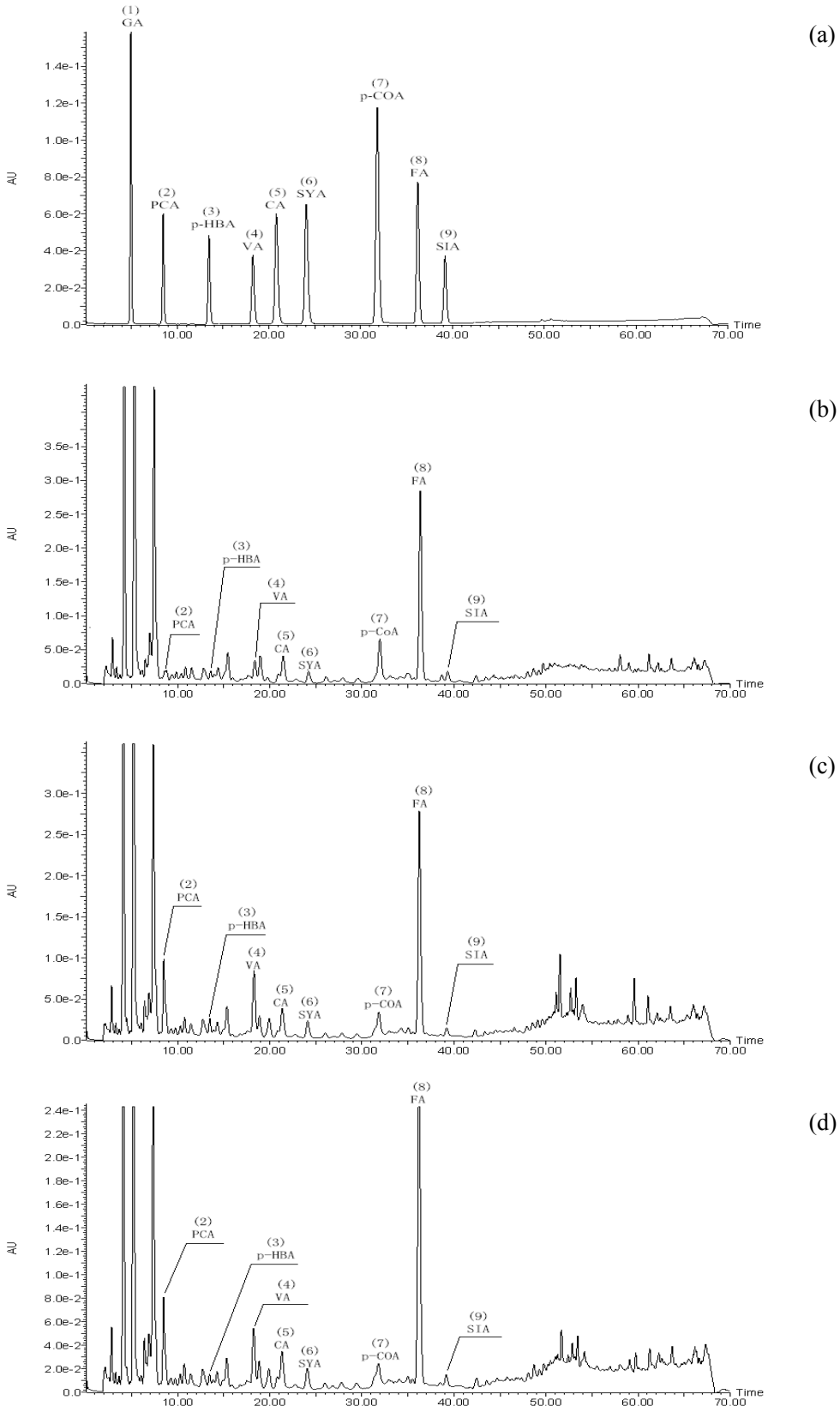


Figure 3.1 HPLC chromatograms of digested phenolic extracts from mixed standard (a) Öelands hvede (b), Indigo (c) and Konini (d) bread samples at 280 nm. Phenolic acids: (1) gallic acid, (2) protocatechuic acid, (3) *p*-hydroxybenzoic acid, (4) vanillic acid, (5) caffeic acid, (6) syringic acid, (7) *p*-coumaric acid, (8) ferulic acid, (9) sinapic acid

Table 3.2 Contents of detected monomeric phenolic acids in digested bread samples

	Öelands hvede	Indigo	Konini
Protocatechuic acid (ug/g)	0.81 ± 0.05 [c]	6.8 ± 0.18 [a]	5.11 ± 0.35 [b]
***p*-Hydroxybenzoic acid (ug/g)**	0.95 ± 0.05 [b]	1.53 ± 0.03 [a]	0.78 ± 0.11 [b]
Vanillic acid (ug/g)	2.03 ± 0.05 [c]	5.51 ± 0.03 [a]	3.51 ± 0.15 [b]
Caffeic acid (ug/g)	0.77 ± 0.03 [b]	0.50 ± 0.03 [a]	0.58 ± 0.07 [b]
Syringic acid (ug/g)	0.92 ± 0.01 [b]	1.14 ± 0.02 [a]	0.99 ± 0.03 [b]
***p*-Coumaric acid (ug/g)**	2.47 ± 0.02 [a]	1.47 ± 0.07 [b]	1.20 ± 0.14 [b]
Ferulic acid (ug/g)	12.14 ± 0.23 [a]	11.75 ± 0.28 [a]	12.27 ± 0.87 [a]
Sinapic acid (ug/g)	1.84 ± 0.00 [a]	1.22 ± 0.07 [b]	1.25 ± 0.06 [b]
Total (ug/g)	21.94 ± 0.45	29.94 ± 0.73	25.69 ± 1.79

[a] Values in each row with different letters are significantly different ($p<0.05$).

The composition of phenolic acids in digested bread extract is summarized in **Table 3.2**. In general, purple wheat varieties contained higher levels of phenolic acids than the control wheat. Correlation analysis demonstrated significant ($p<0.05$) correlations ($r>0.9162$) among total phenolic acid content, TPC, DPPH, ABTS and ORAC. Compared to the control wheat, significantly ($p<0.05$) higher content of protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, syringic acid were detected in Indigo variety; however, *p*-coumaric and sinapic acid were found more concentrated in the control wheat. From the quantitative view, the major phenolic acids were detected in order of ferulic > vanillic > *p*-coumaric > sinapic for all wheat varieties. Protocatechuic acid was found to be present in a remarkably high level in purple wheat, but detected in minor amounts in Öelands hvede. The presence of ferulic, sinapic and *p*-coumaric acid was detected in extracts of *in-vitro* digested whole wheat bread by Hiller et al. (2011), ranging from 1.0–2.6, 0.1-0.4, 0.5-1.9 ug/mL, respectively. Hemery et al (2010) reported values of 0.28, 1.68 and 8.2 ug/g for free *p*-coumaric, sinapic and ferulic acid, respectively in *in-vitro* digested whole wheat bread. It seems likely that the unique phenolic profile of purple wheat led to the variations in detected amounts of phenolic acids compared to values reported in the literature.

Phenolic acid profiles were previously summarized in **Table 2.3** (free) and **2.4** (bound). *p*-Hydroxybenzoic, vanillic, *p*-coumaric and ferulic acid were detected in free- while protocatechuic, caffeic syringic and sinapic were additional acids in bound-fraction. *In-vitro* GI tract model was expected to partially release some bound phenolic acids, since all eight phenolic acids were present in digested extract. However, an incomplete extraction of soluble free phenolic acids was observed as reflected by the lower levels of *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acid that were detected in the digested extracts.

3.4.4. Cytotoxicity of Digested Bread Sample in Different Cell Lines

Figure 3.2 (a) shows the absorbance of formed formazan decomposed from MTT reagent. The higher absorbance reflected the higher degree of living cells. For the HT-29 cell line, no toxic effect was observed when treatment was applied from 5 to 100 mg/mL. However, when the concentration rose up to 500 mg/mL, significant ($p<0.05$) decrease in numbers of living cells was detected. Constant levels of living cells were obtained for Caco-2 cell line, when extracts were applied at 0 to 20 mg/mL. Increase in cell numbers occurred at extract concentration of 100 and 500 mg/mL. For FHs cell line, more cells were alive at lower extract concentrations (5 and 20 mg/mL). Decrease in numbers of living cells was observed at 100 and 500 mg/mL.

Figure 3.2 (b) shows the cytotoxicity of sample treatment at different concentrations of 5, 20, 100 and 500 mg/mL. Cell viability was calculated by referring the absorbance of sample treatment to the untreated control (control had 100% viability). As seen in **Figure 3.2 (b)**, different cell lines had varied responses to the same environmental condition. In HT-29

cell line, cell viability ranged from 83% to 101% and 104% to 136% at concentrations of 5 and 100 mg/mL. However, loss of around 60% cells was observed when treatment increased to 500 mg/mL. For Caco-2 cell line, when concentration increased from 5 to 500 mg/mL, the cell viability ranged from 105% to 154%, 107% to 162% and 104% to 152% for Öelands hvede, Indigo and Konini, respectively. Meanwhile, the cell viability of FHs cell line ranged from 73% to 228%, 96% to 257% and 76% to 243% for Öelands hvede, Indigo and Konini, respectively. Therefore, except for 500 mg/mL for HT-29 cell line, other concentrations were considered to pose no toxicity against the three cell lines.

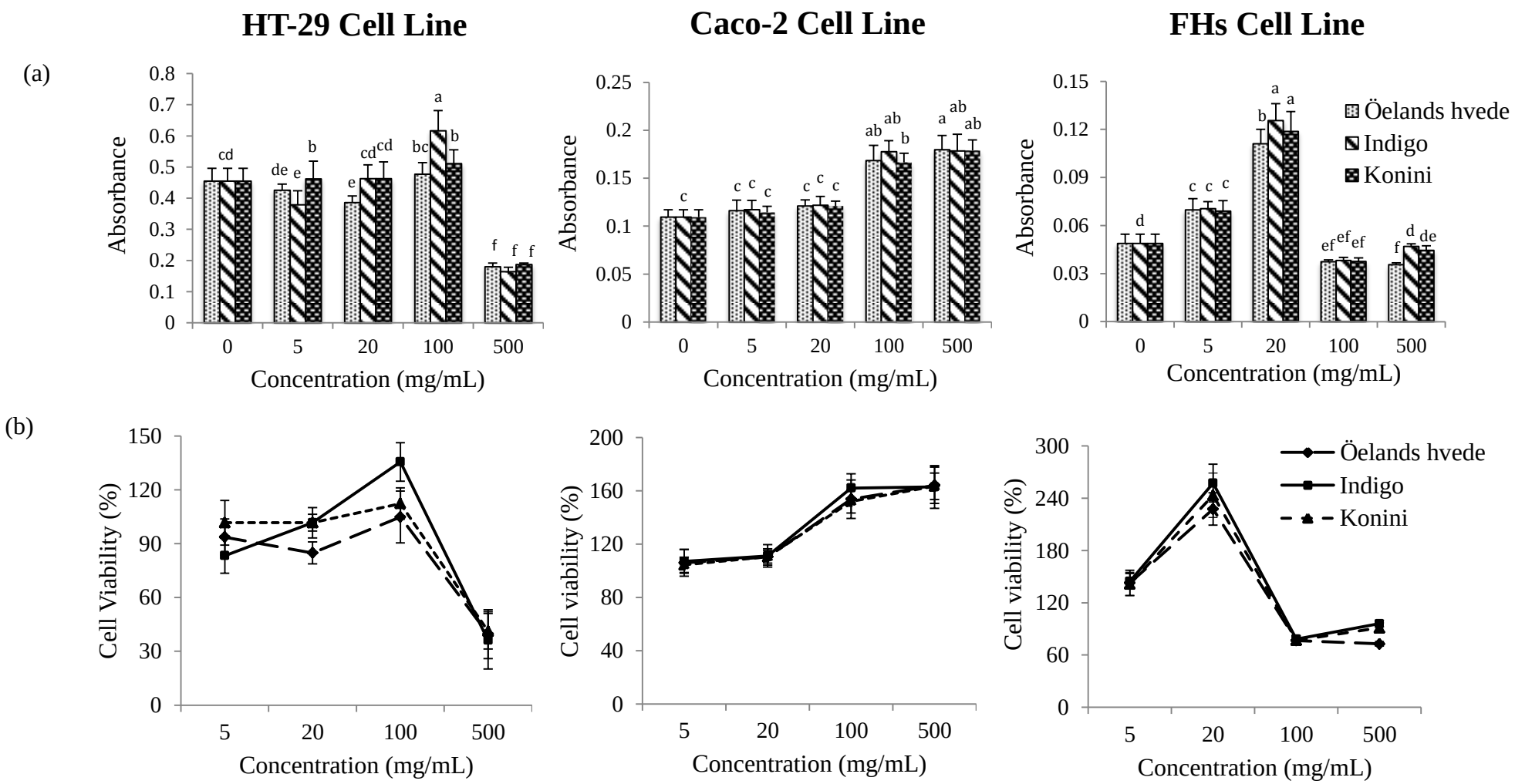


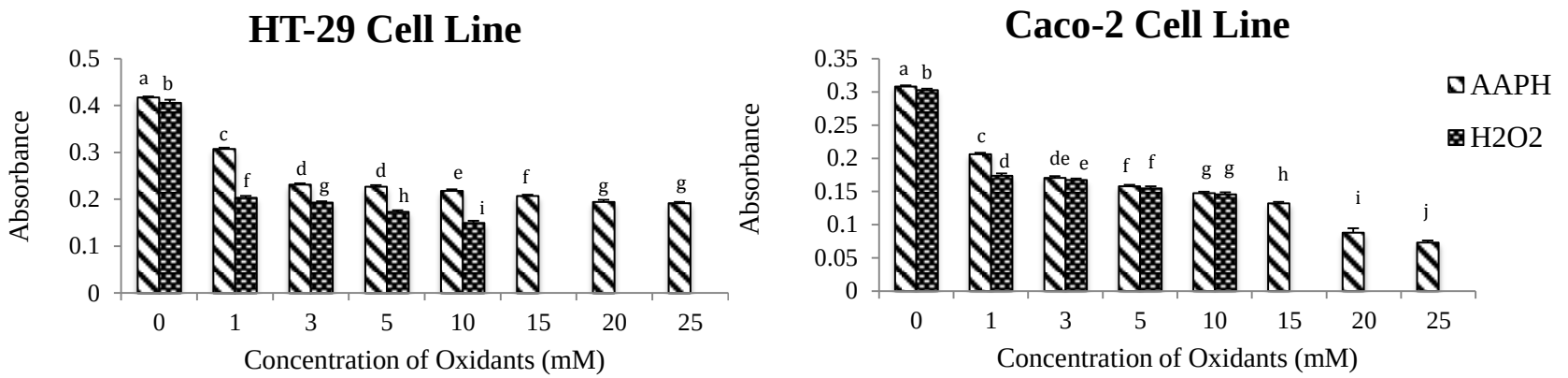
Figure 3.2 Absorbance (a) and viability (b) of cells treated with different concentrations of digested bread extracts in different cell lines measured using the MTT assay. Results were expressed as mean ± SD. Values labelled with different letters for different cell lines are significantly different ($p<0.05$).

3.4.5. Cytotoxicity of Various Concentrations of AAPH and H_2O_2

A preliminary cytotoxic effect of different concentrations of AAPH and H_2O_2 on HT-29 and Caco-2 cell lines was conducted to determine the optimal concentrations of AAPH and H_2O_2 to induce cellular oxidative stress.

Figure 3.3 summarizes the toxicity of AAPH and H_2O_2. The increase in concentrations resulted in increase of cell loss for both lines. Following AAPH oxidative stress, the loss of cell viability was observed in ranges of 26% to 56% and 33% to 76% in HT-29 and Caco-2 cell lines, respectively. Meanwhile, with the treatment of H_2O_2, cell viability ranged from 37% to 50% and 48% to 57% for HT-29 and Caco-2 cell lines, respectively. As seen in **Figure 3.3 (b)**, the major decline in HT-29 cell viability was found at concentrations of 3 mM AAPH and 1 mM H_2O_2, leading to 45% and 50% of cell death, respectively. The cell viability maintained at a relatively constant level when higher concentrations were applied. For Caco-2 cell line, when the levels of AAPH and H_2O_2 increased to 3 mM and 1 mM, the loss of cell viability went up to approximately 45%, yielding a cell viability of 55%. Thus, 3 mM of AAPH and 1 mM of H_2O_2 were chosen to stimulate sufficient levels of oxidation in the subsequent studies. Since the non-transformed cells (FHs) are more sensitive to oxidative stressors compared to the cancer cells, the selected concentrations (3 mM of AAPH and 1 mM of H_2O_2) are considered to exert toxic effects on FHs cell line.

(a)



(b)

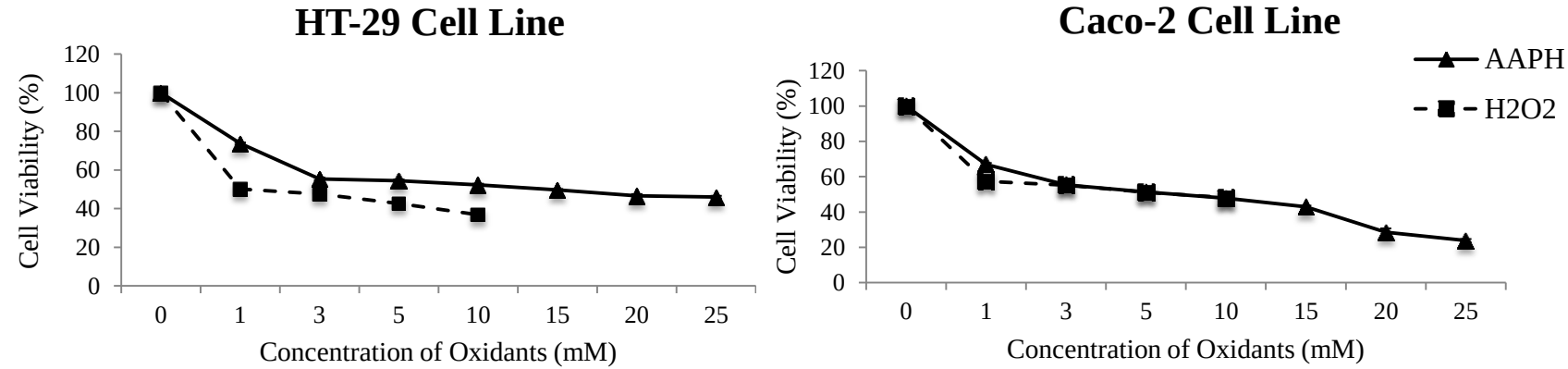


Figure 3.3 Absorbance (a) and viability (b) of cells treated with different concentrations of AAPH and H_2O_2 in different cell lines measured using MTT assay. Values labelled with different letters for each cell line are significantly different ($p<0.05$). Results were expressed as mean ± SD.

3.4.6. Cytoprotective Activity of Digested Bread Extract against AAPH-Induced Oxidation

Figure 3.4 shows cytoprotectivity of digested bread extracts against AAPH-induced oxidation. In **Figure 3.4 (b)**, no inhibitory effect was observed for HT-29 cell line. The reduction of cell viability respectively ranged from 10% to 19%, 2% to 9% and 7% to 20% for Öelands hvede, Indigo and Konini with concentrations varying from 5 to 100 mg/mL. Result for 500 mg/mL was not taken into consideration, since it was regarded to be toxic to living cells. In Caco-2 cell line, the inhibitory effect was significantly ($p<0.05$) seen when concentration rose up to 100 mg/mL. Using 5 to 500 mg/mL of sample treatments, cell viability varied from 84% to 95%, 95% to 121% and 86% to 111% for Öelands hvede, Indigo and Konini, respectively. The purple wheat – Indigo exhibited better cytoprotectivity with the highest inhibitory property at concentration of 100 mg/mL. In contrast, FHs cell line exhibited a more pronounced growth at lower concentrations, reaching cell viability of 102%, 139% and 125% at 5 mg/mL and 118%, 148% and 131% at 20 mg/mL for Öelands hvede, Indigo and Konini, respectively. Nevertheless, with the increase in sample concentration, cell viability was suppressed from 25% to 30%.

Specific concentrations of digested bread extracts displayed effective protection in Caco-2 and FHs cells against AAPH-induced cell loss. However, sample treatment demonstrated pro-oxidant effect throughout all the concentrations in HT-29 cells (cell viability < 100%). Some phenolic acids have been reported as pro-oxidants in various evaluation systems, although their antioxidant properties are clear *in-vitro* (Decker, 1997; Fukumoto & Mazza, 2000; Maurya & Devasagayam, 2010). The anti- or pro-oxidant activity of phenolic compounds ultimately depends on sample concentrations and cultural cell lines.

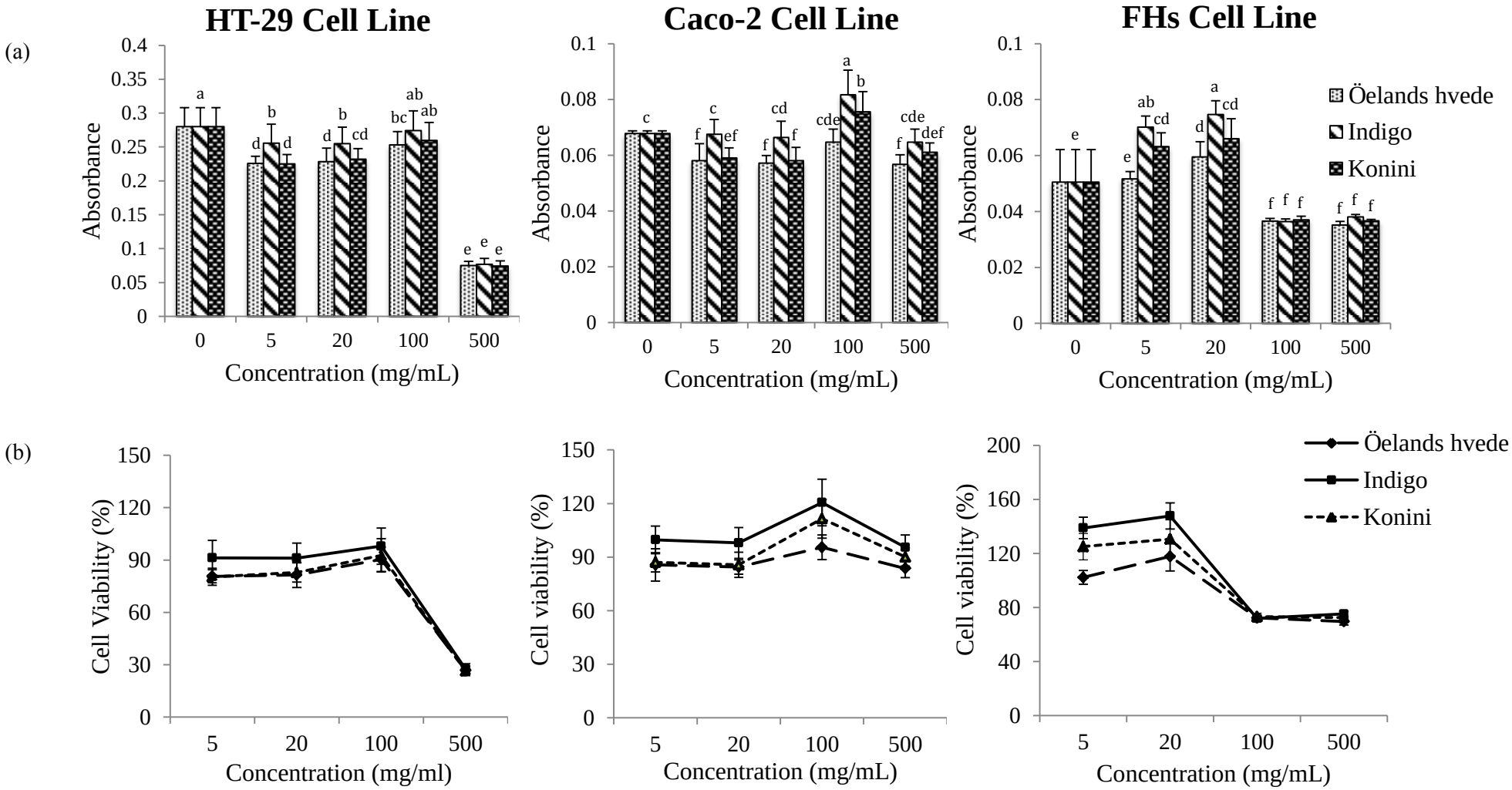


Figure 3.4 Cytoprotective activities of in-vitro digested bread extracts at different concentrations against AAPH-induced oxidation. Value labelled with different letters are significantly different ($p<0.05$). Results were expressed as mean ± SD.

3.4.7. Cytoprotective Activity against H_2O_2-Induced Oxidation

Figure 3.5 shows the cytoprotective activity of sample treatment against H_2O_2-induced oxidation. Digested bread extract started to protect against cell death when the concentration reached to 100 mg/mL in HT-29 cell line. The cell viability reached 85%, 124% and 105% for Öelands hvede, Indigo and Konini, respectively. For Caco-2 cells, cell viability increased with increase in sample concentrations. However, when using 500 mg/mL, bread extracts behaved as pro-oxidants, causing oxidative damage to living cells. The cell loss reached 40%, 25% and 33% for Öelands hvede, Indigo and Konini, respectively. This indicated a dose-dependent manner of sample treatment. A similar phenomenon was observed in FHs cells; whereas, significant cell loss (approximately 40%) was obtained at lower concentration (100 mg/mL). The different responses of cultural cell lines to sample treatment were ascribed to the specific cellular metabolism and subsequent proliferation of different cell types. Purple wheat demonstrated higher cytoprotective activity than the common wheat, confirming its beneficial potential in cellular biological environment, challenged in this case with free radicals.

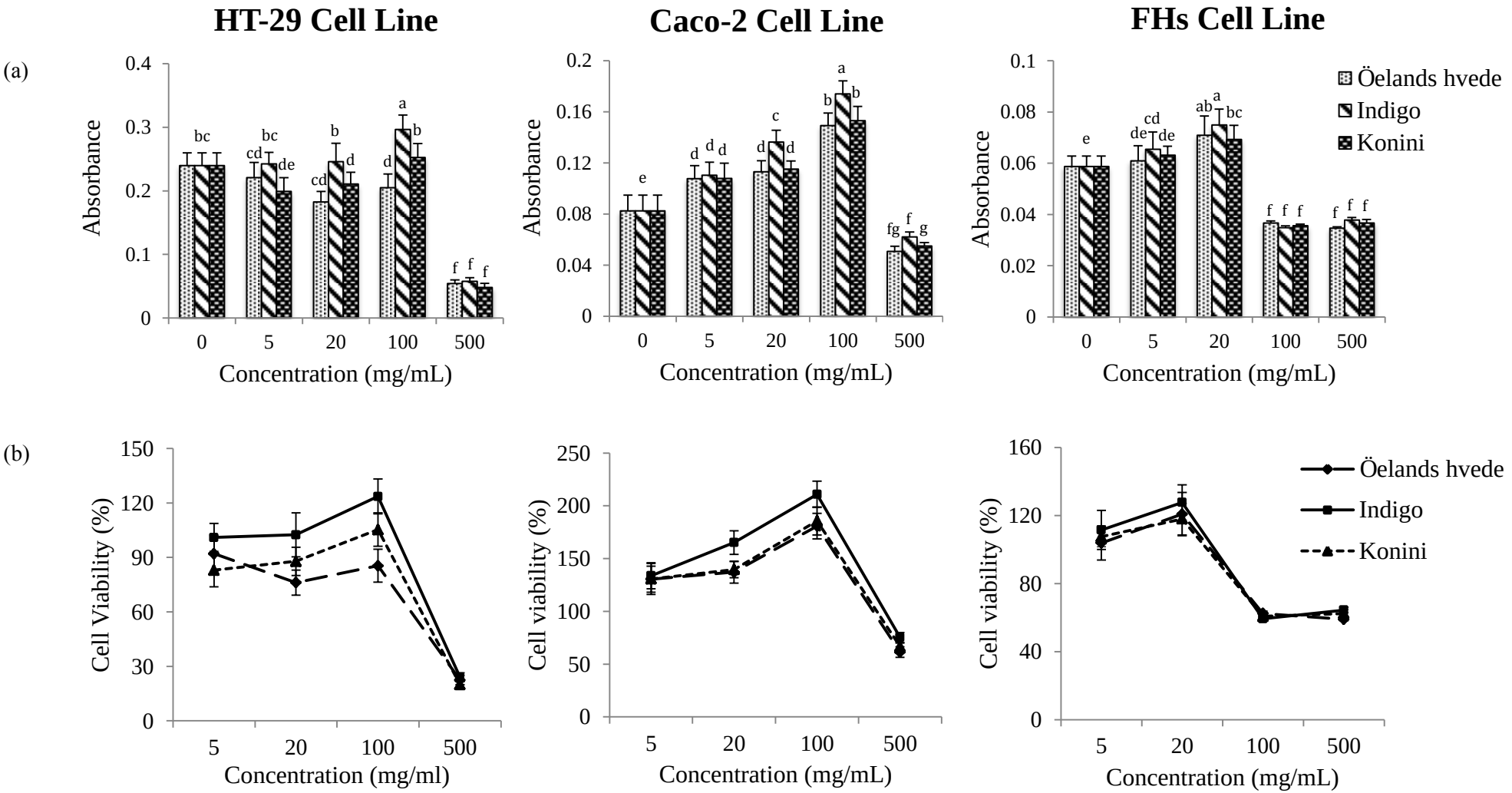


Figure 3.5 Cytoprotective activities of in-vitro digested bread extracts at different concentrations against H2O2-induced oxidation. Values labelled with different letters in different cell lines are significantly different ($p<0.05$). Results were expressed as mean ± SD.

3.4.8. Cellular Antioxidant Activity (CCA)

Figure 3.6 shows the cellular antioxidant activity of sample treatment against AAPH-induced oxidation. The fluorescent intensity represents the degree of oxidation. Positive and negative controls showed the intensity with and without the addition of AAPH. They were used to illustrate how efficiently the AAPH could cause the cellular oxidation. In the presence of antioxidant sample treatment, the oxidation would be inhibited, ideally falling into the range of fluorescence levels between positive and negative controls. As seen in **Figure 3.6**, concentration levels of 5 and 20 mg/mL for HT-29 and Caco-2 cell lines as well as 500 mg/mL for FHs cell line were detected to be higher than negative controls while lower than positive controls over time. Therefore, these concentrations were used to construct the chart (**Figure 3.7a**) for cellular antioxidant activity.

Image 2 shows the fluorescent intensity of HT-29 cells exposed to AAPH-induced oxidation. Indigo at concentration of 5 mg/mL was chosen to display the inhibitory effect of digested extract. **Image 2 (a)** showed the intensity level of cells incubated with sample treatment. The fluorescence gradually increased and finally maintained at a constant level. **Image 2b** exhibited the positive control. Even though the initial fluorescence was relatively lower than that of **Image 2 (a)**, the fluorescence intensity increased rapidly and became higher from 30 min of incubation time. This thereby indicated the ability of digested bread extract to reduce AAPH-induced cellular oxidation.

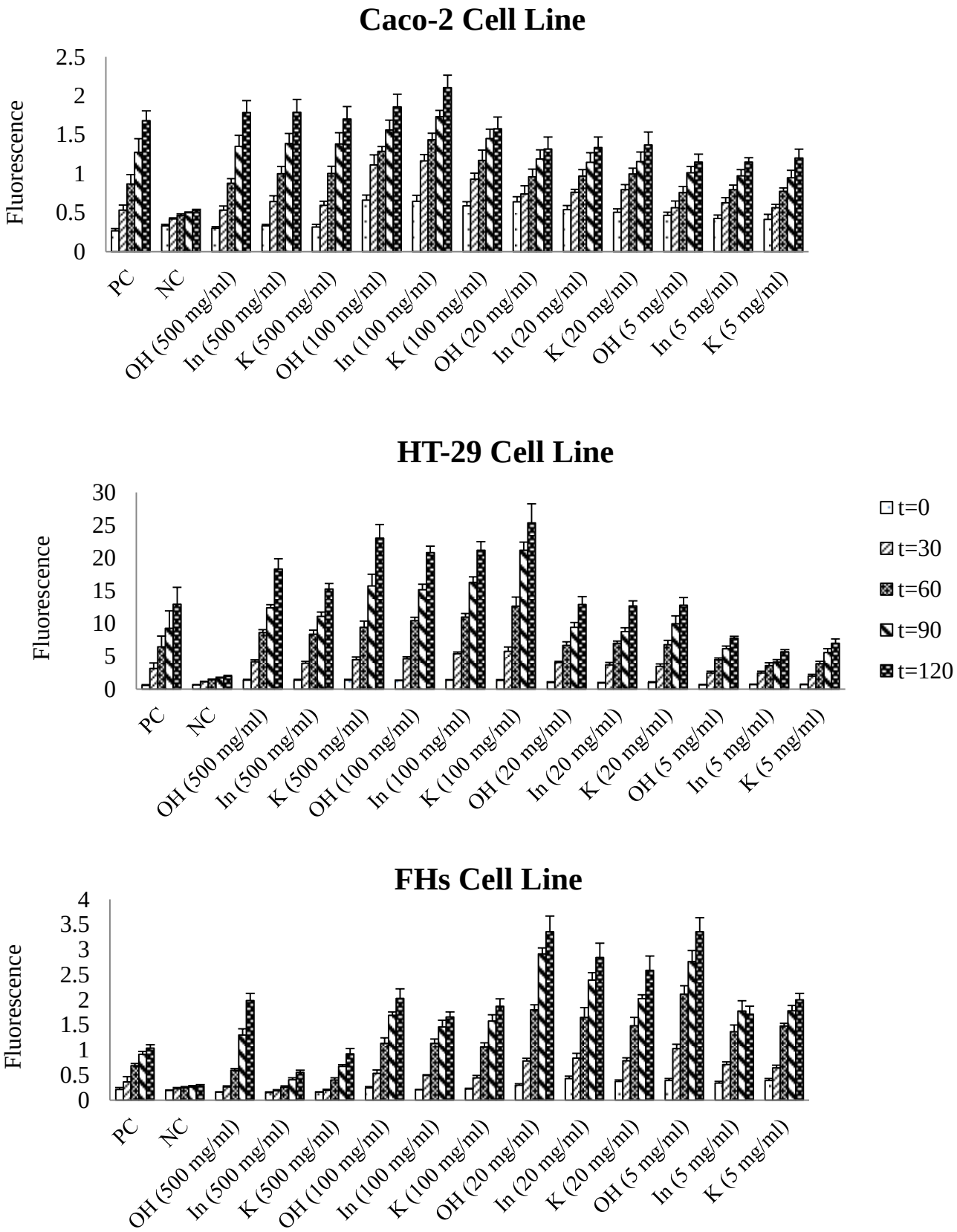
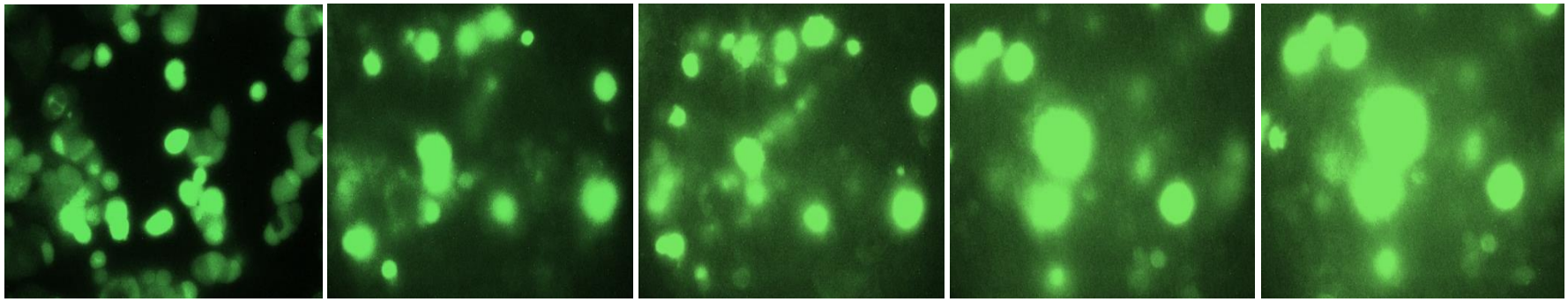


Figure 3.6 Fluorescence intensity (mean ± SD) of AAPH-induced oxidation of DCFH to DCF in different cells lines treated with various concentrations of digested extracts. PC: control with AAPH, NC: control without AAPH, OH: Öelands hvede, In: Indigo, K: Konini

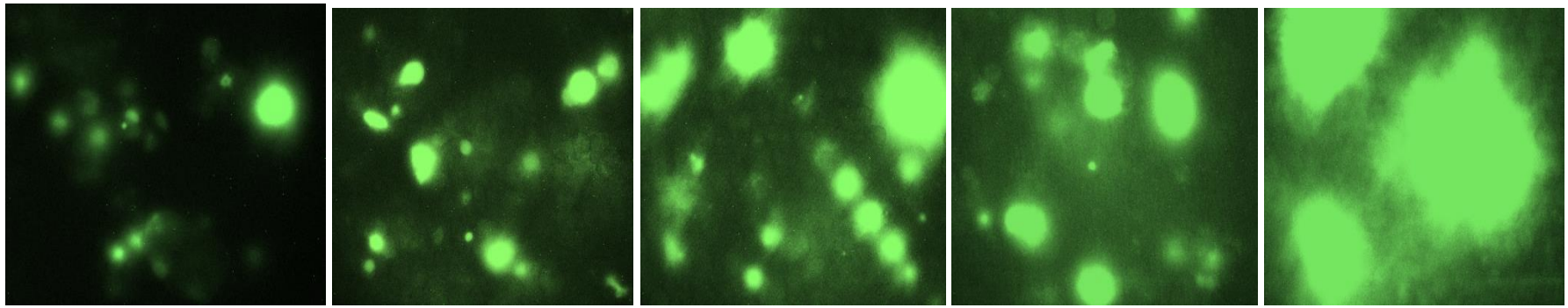
Image 2. Fluorescence intensity of AAPH-induced oxidation with (a) and without (b) addition of Indigo bread extract (5 mg/mL) for cellular antioxidant activity assay at different incubation time in Caco-2 cell line

(a)



t = 0 min | t = 30 min | t = 60 min | t = 90 min | t = 120 min

(b)



t = 0 min | t = 30 min | t = 60 min | t = 90 min | t = 120 min

AAPH initiated intracellular oxidation in these three cell lines as seen in **Figure 3.7 (a)**.The positive control showed a rapid increase in fluorescence level, and the intensity increased over time as compared to the negative control. The AAPH-induced oxidation of dye DCFH to DCF was suppressed by applying sample treatment. The fluorescence intensity was lower than the positive control when cells were incubated with sample treatment except for Öelands hvede extract in FHs cell line. This inhibitory effect was particularly emphasized at longer incubation time.

CCA was calculated by referring the fluorescence intensity of sample and AAPH treated cells to the reversed basis of positive control. The %CCA ranged from -19% to 56% for HT-29 cell line. As seen in **Figure 3.7 (b)**, the sample treatment did not show effect until at an incubation time of 15 min. Then the CCA increased gradually with time. Indigo bread showed the highest antioxidant activity, whereas Öelands hvede extract had the lowest %CCA. Moreover, the increase in CCA was more rapid in Indigo treated cells than the others. A similar phenomenon was obtained for Caco-2 cell line, however, the inhibitory effect was not observed until 45 min. The %CCA ranged from 13% to 32% during the time of 60 to 120 min. With respect to FHs cells, a higher dose (500 mg/mL) of sample treatment was required to exert cellular beneficial property. Opposite to the cancer cell lines, the CCA of normal cell line increased initially and decreased with the increase in incubation time. The preliminary %CCA of FHs cells was 24%, 28% and 25% for Öelands hvede, Indigo and Konini, respectively. The highest %CCA reached 62%, 46% and 27% for Indigo, Konini and Öelands hvede respectively, demonstrating the better antioxidant activity of digested bread extracts from purple wheat.

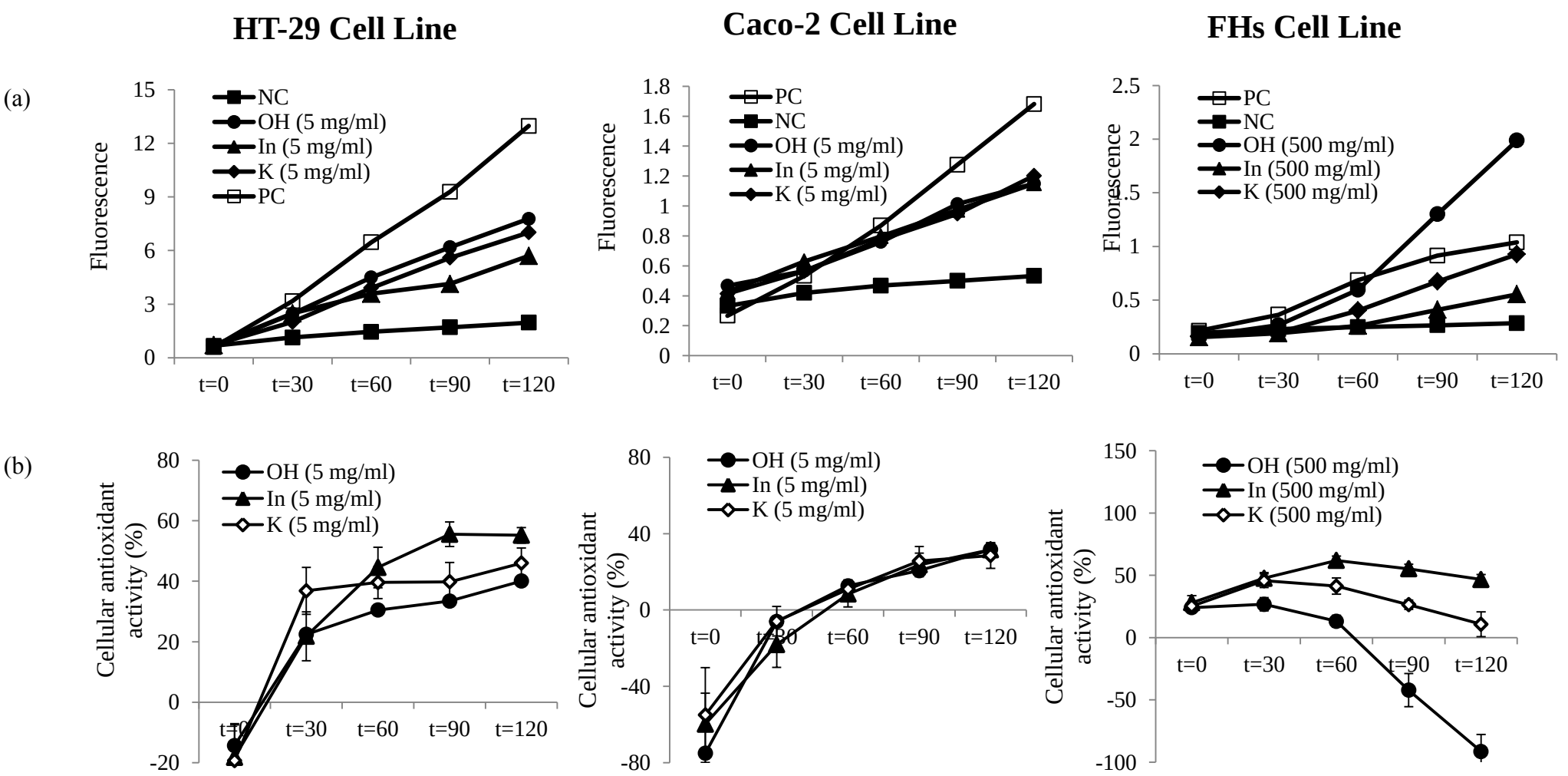


Figure 3.7 Inhibitory effects of certain concentrations of digested bread extracts against AAPH-induced oxidation of DCFH to DCF represented by fluorescence intensity (a) and cellular antioxidant activity (b). PC: positive control with AAPH; NC: negative control without AAPH. Results were expressed as mean ± SD.

3.5. Conclusion

The antioxidant property and phenolic acid profile of purple wheat bread were investigated using *in-vitro* GI tract model. Purple wheat demonstrated significantly ($p<0.05$) higher antioxidant activity than normal wheat. To date, the cytoprotective and cellular antioxidant activity were evaluated for the first time *in-vitro* using digested baked products from purple wheat. However, the cell culture assays exhibited a concentration-dependent manner and the use of different cell lines revealed different cellular antioxidant and pro-oxidant properties. Findings suggest that *in-vitro* digested extracts of purple wheat bread might function to reduce levels of cellular ROS and prevent the formation of apoptotic cells caused by AAPH oxidation.

GERNERAL DISCUSSION

The changes in content of free phenolics (FPC), bound phenolics (BPC) and total anthocyanins (TAC) were investigated for purple wheat varieties during the production of bread. Antioxidant activities (AOA) were examined using DPPH and ABTS assays. Phenolic acids were also identified and quantified using HPLC in samples obtained from selected steps of bread-making. The antioxidant properties and phenolic acid profiles of purple wheat bread were subsequently investigated using an *in-vitro* gastrointestinal (GI) tract model. The digested extracts were examined against total phenolic content (TPC), DPPH values, ABTS levels and phenolic acid profiles using HPLC. Cell culture models were employed for investigating their intracellular antioxidant activities.

Mixing, fermenting and baking significantly ($p<0.05$) increased FPC and BPC. Bread crust contained the highest while bread crumb contained the lowest FPC. BPC exhibited a revisal of result. AOA followed the trends of their respective phenolic contents. HPLC analysis detected four phenolic acids for free and eight phenolic acid for bound fractions. Free phenolic acids were thermally labile to heat. However, different bound phenolic acids showed varying responses to the bread-making process. TAC was significantly ($p<0.05$) reduced through mixing and baking, but fermentation elevated the levels of anthocyanins. Anthocyanin extract of purple wheat exerted higher AOA than these of common wheat. Purple wheat bread extracts after i*n-vitro* gastrointestinal digestion demonstrated significantly ($p<0.05$) higher antioxidant activity than common wheat by using chemical assays. Cytoprotective and cellular antioxidant activities were employed to analyze their cellular functional properties. During *in-vitro* testing, extracts exhibited concentration-dependent effects. Moreover, the use of different cell lines exhibited varying levels of cellular antioxidant and pro-oxidant properties. However, purple wheat demonstrated higher cytoprotective and cellular antioxidant effects than these of common wheat. Our findings

suggest that purple wheat has the potential to act as functional food to scavenge free radicals and reduce cellular oxidative stress. For future studies, more purple wheat varieties with higher TAC can be involved, while animal or human trails are suggested to give more information in *in-vivo* analysis.

The strengths of the present study were: (1) systematic investigation of phenolic changes during bread production from purple wheat, (2) utilization of an *in-vitro* digestion model in combination with cell culture assays, (3) the use of a combination of cancer cell lines and non-transformed (normal) cells. However, weaknesses also appeared. Only one anthocyanin compound, cyanidin-3-glucoside, was detected in wheat varieties. Therefore, it was not worth quantifying the changes in anthocyanin profiles during bread-making process. Also, the non-transformed cell line (FHs) was obtained from the intestine of an infant, which turned out not to be the best model for investigating intracellular antioxidant activities of digested bread extracts.

REFERENCES

AACC International Approved Method. (1999). Moisture-Air oven method. Approved Methods of Analysis, 11th Ed. Official Method 44-15.02. AACC International: St. Paul, MN.

AACC International Approved Method. (2000). Ash-Basic method. Approved Methods of Analysis, 11th Ed. Official Method 08-01.01. AACC International: St. Paul, MN.

Abdel-Aal, E.S.M., Abou-Arab, A.A., Gamel, T.H., Hucl, P., Young, J.C. and Rabalski, I. (2008). Fractionation of blue wheat anthocyanin compounds and their contribution to antioxidant properties. *Journal of Agricultural and Food Chemistry, 56,* 11171-11177.

Abdel-Aal, E.S.M. and Hucl, P. (2003). Composition and stability of anthocyanins in blue-grained wheat. *Journal of Agricultural and Food Chemistry, 51,* 2174–2180.

Abdel-Aal, E.S.M., Young, J.C. and Rabalski, I. (2006). Anthocyanin composition in black, blue, pink, purple, and red cereal grains. *Journal of Agricultural and Food Chemistry, 54,* 4696–4704.

Adom K.K., Sorrells M.E., Liu R.H. (2003) Phytochemical profiles and antioxidant activity of wheat varieties. *Journal of Agricultural and Food Chemistry, 51,* 7825–7834

Adom, K.K., Sorrells, M.E. and Liu, R.H. (2005). Phytochemicals and antioxidant activity of milled fractions of different wheat varieties. *Journal of Agricultural and Food Science, 53,* 2297-2306.

Almajano M.P., Delgado M.E. and Gordon M.H. (2007). Changes in the antioxidant properties of protein solutions in the presence of epigallocatechin gallate. *Food Chemistry, 101,* 126–30.

Alvarez-Jubete, L., Wijngaard, H., Arendt, E.K. and Gallagher, E. (2010). Polyphenol composition and "in vitro" antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. *Food Chemistry, 119,* 770-778.

Andersson, A.A.M., Kamal-Eldin, A. and Aman, P. (2010). Effects of environment and variety on alkylresorcinols in wheat in the HEALTHGRAIN diversity screen. *Journal of Agricultural and Food Chemistry, 58,* 9299-9305.

Angioloni, A. and Collar, C. (2011). Polyphenol composition and "in vitro" antiradical activity of single and multigrain breads. *Journal of Cereal Science, 53,* 90-96.

American Type Culture Collection (ATCC). Information from: http://www.atcc.org. (accessed by August, 2014).

Babich, H., Liebling, E.J., Burger, R.F., Zuckerbraun, H.L. and Schuck, A.G. (2009). Choice of DMEM, formulated with or without pyruvate, plays an important role in assessing the in vitro cytotoxicity of oxidants and pro-oxidant nutraceuticals. *In Vitro Cell*

Developmental Biology, *45,* 226-233.

Bass, D.A., Parce, J.W., Dechatelet, L.R., Szejda, P., Seeds, M.C. and Thomas, M. (1983). Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. *Journal of Immunology, 130,* 1910-1917.

Bast, A., Haenen, G.R. and Doelman, C.J. (1991). Oxidants and antioxidants: state of the art. *Americal Journal of Medicine, 91,* 2S–13S.

Beta, T., Nam, S., Dexter, J.E. and Sapirstein, H.D. (2005). Phenolic content and antioxidant activity of pearled wheat and roller-milled fractions. *Cereal Chemistry, 82,* 390-393.

Bellido, G.G. and Beta, T. (2009). Anthocyanin composition and oxygen radical scavenging capacity (ORAC) of milled and pearled purple, black and common barley. *Journal of Agricultural and Food Chemistry, 57,* 1022-1028.

Bicudo, M.O.P., Ribani, R.H. and Beta, T. (2014). Anthocyanins, phenolic acids and antioxidant properties of juçara fruits (Euterpe edulis M.) along the on-tree ripening process. *Plant Food for Human Nutrition, 69,* 142-147.

Borowicki, A., Stein, K., Scharlau, D., Scheu, K., Brenner-Weiss, G., Obst, U., Hollmann, J., Lindhauer, M., Wachter, N. and Glei, M. (2010). Fermented wheat aleurone inhibits growth and induces apoptosis in human HT29 colon adenocarcinoma cells. *British Journal of Nutrition, 103,* 360-369.

Borrelli RC, Mennella C, Barba F, Russo M, Russo GL, Krome K, Erbersdobler HF, Faist V, Fogliano V. (2003). Characterization of coloured compounds obtained by enzymatic extraction of bakery products. *Food Chemistry and Toxicology, 41,* 1367–74.

Bueno, J.M., Ramos-Escudero, F., Saez-Plaza, P., Munoz, A.M., Navas, M.J. and Asuero, A. (2012). Analysis and antioxidant capacity of anthocyanin pigments. Part I: General considerations concerning polyphenols and flavonoids. *Critical Reviews in Analytical Chemistry, 42,*102–125.

Bustos, D.V., Riegel, R. and Calderini, D.F. (2012). Anthocyanin content of grains in purple wheat is affected by grain position, assimilate availability and agronomic management. *Journal of Cereal Science, 55,* 257-264.

Bunzel, M., Ralph, J., Marita, J.M., Hatfeld, R.D. and Steinhart, H. (2001). Diferulates as structural components in soluble and insoluble cereal dietary fibre. *Journal of the Science of Food and Agriculture, 81,* 653-660.

Bunzel, M., Ralph, J., Marita, J.M. and Steinhart, H. (2000). Identification of 4-0-5'- coupled diferulic acid from insoluble cereal fiber. *Journal of Agricultural and Food Chemistry*, *48,* 3166–3169.

Canadian Short Process method. Methods and definitions. In Quality of western Canadian wheat exports (pp. 22–26). 1 February 1997 to 31 July 1997. Grain Research Laboratory, Canadian Grain Commission, Winnipeg, MB.

Challacombe, C.A., Abdel, E.M., Seetharaman, K. and Duizer, L.M. (2012). Influence of phenolic acid content on sensory perception of bread and crackers made from red or white wheat. *Journal of Cereal Science, 56,* 181-188.

Chandrasekare, N. and Shahidi, F. (2011). Effect of roasting on phenolic content and antioxidant activities of whole cashew nuts, kernels, and tests. *Journal of Agricultural and Food Chemistry, 59,* 5006-5014.

Chi, H.Y., Lee, C,H., Kim, K.H., Kim, S.L. and Chung, I.M. (2007). Analysis of phenolic compounds and antioxidant activity with H4IIE cells of three different rice grain varieties. *European Food Research and Technology, 225,* 887-893.

Choia, Y., Jeonga, H. and Lee, J. (2007). Antioxidant activity of methanolic extracts from some grains consumed in Korea. *Food Chemistry, 103,* 130–138.

Coles, L. T., Moughan, P. J. and Darragh, A. J. (2005). In vitro digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple stomached animals. *Animal Food Science and Technology, 123–124,* 421–444.

Collins, A.R. (2005). Assays for oxidative stress and antioxidant status: applications to research into the biological effectiveness of polyphenols. *American Journal of Clinical and Nutrition, 81,* 261S-267S.

Decker, E.A. (1997) Phenolics: prooxidants or antioxidants? *Nutrition Reviews, 55,* 396-407.

Dedio, W., Hill, R.D. and Evans, L.E. (1972). Anthocyanins in the pericarp and coleoptiles of purple wheat. *Canadian Journal of Plant Science, 52,* 977–980.

Dykes, L. and Rooney, L.W. (2007). Phenolic compounds in cereal grains and their health benefits. *Cereal Foods World. 52,* 105-111.

Eyoum, A., Celhay, F., Neron, S., El Amrani, F., Poiffait, A., Potus, J., Baret, J.-L., Nicolas, J. (2002). Biochemical factors of importance in the oxygen consumption of unyeasted wheat flours during dough mixing. In: M. Courtin, W.S. Veraverbeke, J. Delcour, (Eds.), *Recent Advances in Enzymes in Grain Processing* (pp. 303-309). Katholieke University Leuven, Leuven.

European Collection of Cell Cultures (ECCC). Information from https://www.phe-culturecollections.org.uk/products/index.aspx. (accessed August, 2014)

Freshney, R.I. (2006). Basic principles of cell culture. In: G. Vunjak-Novakovic and R.I., Freshney (Eds). *Culture of cells for tissue engineering.* Hoboken, New Jersey: John Wiley & Sons, Inc.

Freshney, R.I. (2010). *Culture of animal cells: A manual of basic technique and specialized application*. Hoboken, New Jersey: John Wiley & Sons, Inc.

Fukumoto, L.R. and Mazza, G. (2000). Assessing antioxidant and prooxidant activities of phenolic compounds. *Journal of Agricultural and Food Chemistry, 48,* 3597-3604.

Galati, G., Sabzevari, O., Wilson, J.X. and O'Brien, P.J. (2002). Prooxidant activity and cellular effects of the phenoxyl radicals of dietary flavonoids and other polyphenolics. *Toxicology, 177,* 91–104.

Garcia-Conesa, M.T., Wilson, P.D., Plumb, G.W., Ralph, J. and Williamson, G. (1999). Antioxidant properties of 4, 4'-dihydroxy-3, 3'-dimethoxy-*β, β'*-bicinnamic acid (8-8-diferulic acid, non-cyclic form). *Journal of the Science of Food and Agriculture, 79,* 379-384.

Garcia-Salas, P., Morales-Soto, A., Segura-Carretero, A. and Fernandez-Gutierrez, A. (2010). Phenolic-compound-extraction systems for fruit and vegetable samples. *Melecules, 15,* 8813-8826.

Gawlik-Dziki, U., Dziki, D., Baraniak, B. and Lin, R. (2009). The effect of simulated digestion in vitro on bioactivity of wheat bread with Tartary buckwheat flavones addition. *Food Science and Technology, 42,* 137-143.

Gelinas, P. and McKinnon, C.M. (2006). Effect of wheat variety, farming site, and bread-baking on total phenolics. *International Journal of Food Science and Technology. 41*, 329-332.

Gliwa, J., Gunenc, A., Ames, N., Willmore, W.G. and Hosseinian, F.S. (2011) Antioxidant activity of alkylresorcinols from rye bran and their protective effects on cell viability of PC-12 AC cells. *Journal of Agricultural and Food Chemistry, 59,* 11473-11482.

Giusti, M.M. and Wrolstad, R.E. (2005). Chariacterization and measurement of anthocyanins by UV-visible spectroscopy. In: *Handbook of Food Analytical Chemistry Pigments, Colorant, Flavors, Textrure, and Bioactive Food Components*. R.E. Wrolstad, T.E. Acree, E.A. Decker, M. Penner, D.S. Reid, S.J. Schwartz, C.F. Shoemaker, D. Smith, and P. Sporns (Eds). Wiley Interscience, Hoboken, NJ, USA.

Guo, W. and Beta, T. (2013). Phenolic acid composition and antioxidant potential of insoluble and soluble dietary fibre extracts derived from selected whole-grain cereals. *Food Research International, 51,* 518-525.

Gurierrez-Uribe, J.A., Romo-Lopez, I. and Serna-Saldivar, S.O. (2011). Phenolic composition and mammary cancer cell inhibition of extracts of whole cowpeas (Vigna unguiculata) and its anatomical parts. *Journal of Functional Foods, 3,* 290-297.

Halliwell, B. (2008). Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? *Archives of Biochemistry and Biophysics, 476,* 107-112.

Halliwell, B. and Whiteman, W. (2004). Measuring reactive species and oxidative damage in vivo and in cell culture: How should you do it and what do the results mean? *British Journal of Pharmacology, 142,* 231-255.

Han, H.M. and Koh, B.K. (2011). Antioxidant activity of hard wheat flour, dough and bread prepared using various processes with the addition of different phenollic acids. *Journal of the Science of Food and Agriculture. 91,* 604-608.

Hansen, H.B., Andreasen, M.F., Nielsen, M.M., Larsen, L.M., Knudsen, K.E.B., Meyer, A.S., Christensen, L.P. and Hansen, A. (2002). Changes in dietary fibre, phenolic acids and activity of endogenous enzymes during rye bread-making. *European Food Research and Technology 214,* 33-42.

Haslam, E. (2007). Vegetable Tannins—Lessons of a Phytochemical Lifetime. *Phytochemistry, 68* , 2713–2721.

Hatcher, D. and Kruger, J. (1997). Simple phenolics acids in flours prepared from Canadian wheat: relationship to ash content, colour, and polyphenol oxidase activity. *Cereal Chemistry*, *74,* 337-347.

He, J. and Giusti, M.M. (2010). Anthocyanins: Natural colourants with health-promoting properties. *Annual Review of Food Science Technology, 1,* 163-187.

Hemery, Y.M., Anson, N.M., Havenaar, R., Haenen, G.R.M.M., Noort, M.W.J. and Rouau, X. (2010). Dry-fractionation of wheat bran increases the bioaccessibility of phenolic acids in breads made from processed bran fractions. *Food Research International, 43,* 1429-1438.

Hiller, B., Schlormann, W., Glei, M. and Lindhauer, M.G. (2011). Comparative study of colorectal health related compounds in different types of bread: Analysis of bread samples pre and post digestion in a batch fermentation model of the human intestine. *Food Chemistry, 125,* 1202-1212.

Hirawan, R., Diehl-Jones, W. and Beta, T. (2011). Comparative evaluation of the antioxidant potential of infant cereals produced from purple wheat and red rice grains and LC-MS analysis of their anthocyanins. *Journal of Agricultural and Food Chemistry, 59,* 12330-12341.

Hosseinian, F. S. and Beta, T. (2007). Saskatoon and wild blueberries have higher anthocyanin contents than other Manitoba Berries. *Journal of Agricultural and Food Chemistry, 55*, 10832–10838.

Hosseinian, F.S., Li, W. and Beta, T. (2008). Measurement of anthocyanins and other phytochemicals in purple wheat. *Food Chemistry, 109,* 916-924.

Hu, C., Cai, Y.Z., Li, W., Corke, H. and Kitts, D.D. (2007) Anthocyanin characterization and bioactivity assessment of a dark blue grained wheat (*Triticum aestivum* L. cv. Hedong Wumai) extract. *Food Chemistry, 104,* 955-961.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J. A. and Prior, R. L. (2002). High-throughput assay of oxygen absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *Journal of Agricultural and Food Chemistry, 50,* 4437–4444.

Hur, S.J., Lim.B.O., Decker, E.A. and McClements, D.J. (2011). In vitro human digestion models for food applications. *Food Chemistry, 125,* 1-12.

Irakli, M.N., Samanidou, V.F., Biliaderis, C.G. and Papadoyannis, I.N. (2012). Development and validation of an HPLC-method for determination of free and bound phenolic acids in cereals after solid-phase extraction. *Food Chemistry, 134,* 1624-1632.

Jacobs, D.R., Meyer, K.A., Kushi, L.H. and Folsom, A.R. (1988). Whole grain intake may reduce risk of coronary heart disease death in postmenopausal women: The Iowa women's health study. *American Journal Clinical Nutrition, 68,* 248-257.

Janicke, B., Hegardt, C., Krogh, M., Onning, G., Akesson, B., Cirenajwis, H.M. and Oredsson, S.M. (2011). The Antiproliferative Effect of Dietary Fiber Phenolic Compounds Ferulic Acid and p -Coumaric Acid on the Cell Cycle of Caco-2 Cells. *Nutrition and Cancer, 63,* 611-622.

Jing, P., Noriega, V., Schwartz, S. J. and Giusti, M. (2007). Effects of growing conditions on purple corncob (Zea mays L.) anthocyanins. *Journal of Agricultural and Food Chemistry, 55,* 8625-8629.

Kanski, J., Aksenova, M., Stoyanova, A. and Butterfield, D.A. (2002). Ferulic acid antioxidant protection against hydroxyl and peroxyl radical oxidation in synaptosomal and neuronal cell culture systems *in vitro*: structure-activity studies. *Journal of Nutritional biochemistry, 13,* 273-281.

Katina, K., Arendt, E., Liukkonen, K.H., Autio, K., Flander, L. and Poutanen, K. (2005). Potential of sourdough for healthier cereal products. *Trends in Food Science and Technology, 16,* 104-112.

Kessler, M., Ubeaud, G. and Jung, L. (2003). Anti-and pro-oxidant activity of rutin and quercetin derivatives. *Journal of Pharmacy and Pharmacology, 55*, 131-142.

Keston, A.S. and Brandt, R. (1965). The fluorometric analysis of ultramicro quantities of hydrogen peroxide. *Analytical Biochemistry, 11,* 1-5.

Khanduja, K.L. and Bhardwaj, A. (2003). Stable free radical scavenging and antiperoxidative properties of resveratrol compared in vitro with some other bioflavonoids. *India Journal of Biochemistry and Biophysics, 40,* 416-422.

Kim, K.H., Tsao, R., Yang, R. and Cui, S.W. (2006). Phenolic acid profile and antioxidant activities of wheat bran extracts and the effect of hydrolysis conditions. *Food Chemistry, 95,* 466-473.

Klepacka, J. and Fornal, L. (2006). Ferulic acid and its position among the phenolic compounds of wheat. *Critical Reviews in Food Science and nutrition, 46,* 639-647.

Knievel, D.C., Abdle-Aal, E.S.M., Rabalski, I., Nakamura, T. and Hucl, P. (2009). Grain color development and the inheritance of high anthocyanin blue aleurone and purple pericarp in spring wheat (Triticum aestivum L.). *Journal of Cereal Science, 50,* 113-120.

Kong, J.M., Chia, L.S., Goh, N.K., Chia, T.F. and Brouillard, R. (2003) Analysis and biological activities of anthocyanins. *Phytochemistry, 64,* 923-933.

Labieniec, M., Gabryelak, T. (2007). Antioxidant and oxidative changes in the digestive gland cells of freshwater mussels *Unio tumidus* caused by selected phenolic compounds in the presence of H_2O_2 or Cu^{2+} ions. *Toxicology in Vitro, 21,* 146-156.

Laughton, M.J., Halliwell, B., Evans, P.J., Robin, J. and Hoult S. (1989). Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin: effects of lipid peroxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. *Biochem Pharmacol, 38,* 2859–64.

Lee, J., Durst, R.W. and Wrolstad, R.E. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborative study. *Journal Association of Official Analytical Chemists International, 88*, 1269–1278.

Lee, J., Kim, J., Park, J., Chung, G. and Jang, Y. (2003). The antioxidant, rather than prooxidant, activities of quercetin on normal cells: quercetin protects mouse thymocytes from glucose oxidase-mediated apoptosis. *Experimental Cell Research, 291,* 286-397.

Lee, K. G. and Shibamoto, T. (2002). Toxicology and antioxidant activities of non-enzymatic browning reaction products: Review. *Food Reviews International, 18,* 151–175.

Leenhardt, F., Lyan, B., Rock, E., Boussard, A., Potus, J., Chanliaud, E. and Remesy, C. (2006). Wheat lipoxygenase activity induces greater loss of carotenoids than vitamin E during bread-making. *Journal of Agricultural and Food Chemistry, 54,* 1710-1715.

Lengyel, E., Oprean, L., Iancu, R., Ketney, O. and Tita, O. (2012). Anthocyanins and polyphenols content in red merlot, cabernet sauvignon and pinot noir wines from recas vineyard. *Acta Universitatis Cibiniensis Series E: Food Technology, 14,* 51-56.

Lesschaeve, I. and Noble, A.C. (2005). Polyphenols: factors influencing their sensory properties and their effects on food and beverage preferences. *American Journal of Clinic and Nutrition, 81,* 330S-335S.

Li, W., Pichard, M.D. and Beta, T. (2007). Effect of thermal processing on antioxidant properties of purple wheat bran. *Food Chemistry, 104,* 1080-1086.

Li, W., Shan, F., Sun, S., Corke, H. and Beta, T. (2005). Free radical scavenging properties and phenolic content of Chinese blackgrained wheat. *Journal of Agricultural and Food Chemistry, 53,* 8533–8536.

Lila M.A. (2004). Anthocyanins and human health: an in vitro investigative approach. *Journal of Biomedicine and Biotechnology, 5,* 306-313.

Lin, L.Z. and Harnly, J. M. (2007). A Screening Method for the Identification of Glycosylated Flavonoids and Other Phenolic Compounds Using a Standard Analytical Approach for All Plant Materials. *Journal of Agricultural and Food Chemistry, 55,*

1084–1096.

Liu, L., Zubik, L., Collins, F.W., Marko, M., Meydani, M. and Mohsen, M. (2004). The antiantherogenic potential of oat phenolic compounds. *Atherosclerosis, 175,* 39-49.

Liu, R.H. (2007). Whole grain phytochemicals and health. *Journal of Cereal Science, 46,* 207-219.

Liu, R.H. (2004). Potential synergy of phytochemicals in cancer prevention: mechanism of action. *Journal of Nutrition, 134,* 3479s-3486s.

Liu, Q., Qiu, Y. and Beta, T. (2010). Comparison of antioxidant activities of different colored wheat grains and analysis of phenolic compounds. *Journal of Agricultural and Food Chemistry, 58,* 9235-9241.

Lv, J., Yu, L., Lu, Y., Niu, Y., Liu, L., Costa, J. and Yu, L. (2012). Phytochemical compositions, and antioxidant properties, and antiproliferative activities of wheat flour. *Food Chemistry, 135,* 325-331.

Majoul, T., Bancel, E., Triboi, E., Ben Hamida, J. and Branlard, G. (2003). Proteomic analysis of the effect of heat stress on hexaploid wheat grain: Characterization of heat-responsive proteins from total endosperm. *Prometoics, 3*, 175–183.

Manach, C., Scalbert, A., Morand, C., Remesy, C. and Jimenez, L. (2004). Polyphenols: Food sources and bioavailability. *American Journal of Clinical Nutrition, 79,* 727–747.

Manzocco, L., Calligaris, S., Mastrocola, D., Nicoli, M.C. and Lerici, R. C. (2001). Review of non-enzymatic browning and antioxidant capacity in processed foods. *Trends in Food Science and Technology, 11,* 340-346.

Matsuo, M., Sasaki, N., Saga, K. and Kaneko, T. (2005). Cytotoxicity of flavonoids toward cultured normal human cells. *Biological and Pharmaceutical Bulletin, 28,* 253-259.

Maurya, D.K. & Devasagayam, T.P. (2010) Antioxidant and prooxidant nature of hydroxycinnamic acid derivatives ferulic and caffeic acids. *Food and Chemical Toxicology, 48,* 3369-3373.

McKenna, T. (2009). *Oxidative stress on mammalian cell cultures during recombinant protein expression*. Linkopings University: Liu-Tryck.

Mermelstein, N.H. (2008). Determining antioxidant capacity. *Food Technology, 62,* 63-66.

Metodiewa, D., Jaiswal, A.K., Cenas, N., Dichancaite, E. and Segura-Aguilar, J. (1999). Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. *Free Radical Biology and Medicine, 26,* 107-116.

Michalska, A., Amigop-Benavent, M., Zielinski, H., and del Castillo, M. D. (2008). Effect of bread making on formation of Maillard reaction products contributing to the overall antioxidant activity of rye bread. *Journal of Cereal Science, 48,* 123-132.

Moheb, A., Grondin, M., Ibrahim, R.K., Roy, R.R. and Sarhan, F. (2013). Winter wheat hull (husk) is a valuable source for tricin, a potential selective cytotoxic agent. *Food Chemistry, 138,* 2-3.

Moore, J., Luther, M., Cheng, Z. and Yu, L. (2009). Effects of baking conditions, dough fermentation, and bran particle size on antioxidant properties of whole-wheat pizza crusts. *Journal of Agricultural and Food Chemistry, 57,* 832-839.

Moore, J. and Yu, L. (2008). Methods for antioxidant capacity estimation of wheat and wheat-based food products. In: L. Yu (Eds.). *Wheat antioxidants* (pp.118-166). Hoboken, New Jersey: John Wiley & Sons, Inc.

Movahed, A., Yu, L., Thandapilly, S.J., Louis, X.L. and Netticadan, T. (2012). Resveratrol protects adult cardiomyocytes against oxidative stress mediated cell injury. *Archives of Biochemistry and Biophysics, 527,* 74–80

Munter, J.L., Hu, F.B., Spiegelman, D., Franz, M. and Dam, R.M. (2007). Whole grain, bran and germ intake and risk of type 2 diabetes: a prospective cohort study and systematic review. Public Library of Science Medicine, 4, 1385-1395.

Nie, L., Wise, M.L., Pererson, D.M. and Meydani, M. (2006). Avenanthramide, a polyphenol from oats, inhibits vascular smooth muscle cell proliferation and enhances nitric oxide production. *Atherosclerosis, 186,* 260-266.

Nichenametla, S. N., Taruscio, T. G., Barney, D. L. and Exon, J. H. (2006). A Review of the Effects and Mechanisms of Polyphenolic in Cancer. *Critical Reviews in Food Science and Nutrition, 46,* 161–183.

Okarter, N., Liu, C., Sorrells, M.E. and Liu, R.H. (2010). Phytochemical content and antioxidant activity of six diverse varieties of whole wheat. *Food Chemistry, 199,* 249-257.

Oomen, A. G., Hack, A., Minekus, M., Zeijdner, E., Cornelis, C., Schoeters, G., Verstraete, W., Van de Wiele, T., Wraqq, J., Rompelberq, C.J., Sips, A.J. and Van Wijnen, J.H. (2002). Comparison of five in vitro digestion models to study the bioaccessibility of soil contaminants. *Environmental Science & Technology, 36,* 3326–3334.

Ou, B., Huang, D., Hampsch-Woodill, M., Flanagan, J.A. and Deemer, E.K. (2002). Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. *Journal of Agricultural and Food Chemistry, 50,* 3122-3128.

Palafox-Carlos, H., Ayala-Zavala, J.F. and Gonzalez-Aguilar, G.A. (2011). The role of dietary fiber in the bioaccessibility and bioavailability of fruit and vegetable antioxidants. *Journal of Food Science, 76,* R6-R15.

Pandey, K.B. and Rizvi, S.I. (2009). Plant polyphenols as dietary antioxidants in human health and disease. *Oxidative Medicine and Cellular Longevity, 2,* 270-278.

Parker, M.L., Ng, A. and Waldron, K.W. (2005). The phenolic acid and polysaccharide

composition of cell walls of bran layers of mature wheat (Triticum aestivum L. cv. Avalon) grains. *Journal of Food Science and Agriculture, 85,* 2539-2547.

Perez-Jimenez, J., Diaz-Rubio, M.E., Mesias, M, Morales, F.J. and Saura-Galixto, F. (2014). Evidence for the formation of maillardized insoluble dietary fiber in bread: A specific kind of dietary fiber in thermally processed food. *Food Research International, 55,* 391-396.

Prior, R.L. and Wu, X. (2006). Anthocyanins: Structural characteristics that result in unique metabolic patterns and biological activities. *Free Radical Research, 40,* 1014-1028.

Prochazkova, D., Bousova, I. and Wilhemova, N. (2011). Antioxidant and prooxidant properties of flavonoids. *Fitoterapia, 82,* 513-523.

Qiu, Y., Liu, Q. and Beta, T. (2010). Antioxidant properties of commercial wild rice and analysis of soluble and insoluble phenolic acids. *Food Chemistry, 121,* 140-147.

Quideau, S., Deffieux, D., Couat-Casassus, C. and Pouysegu, L. (2011). Plant polyphenols: Chemical properties, biological anticities, and synthesis. *Angewandte Chemie, 50,* 586-621.

Renard C.M.G.C., Baron, A., Guyot, S. and Drilleau, J.F. (2001). Interactions between apple cell walls and native apple polyphenols: quantification and some consequences. *International Journal of Biological Macromolecules, 29,* 115–25.

Renger, A. and Steinhart, H. (2000). Ferulic acid dehydrodimers as structural elements in cereal dietary fibre. European. *Food Research Technology, 211,* 422–428.

Rondini, L., Peyrat-Maillard, M.N., Marsset-Baglieri, A., Fromentin, G., Durand, P. and Tome, D. (2004). Bound ferulic acid from bran is more bioavailable than the free compound in rat. *Journal of Agricultural and Food Chemistry, 52,* 4338-4343.

Rota, C., Fann, Y.C. and Mason, R.P. (1999). Phenoxyl free radical formation during the oxidation of the fluorescent dye 20, 70-dichlorofluorescein by horseradish peroxidase. Possible conse-quences for oxidative stress measurements. *Journal of Biological Chemistry, 274,* 28161–28168.

Santos, N.A., Cordeiro, A.M.T.M., Damasceno, S.S., Aguiar, R.T., Rosenhaim, R., Carvalho Filho, J.R., Santos, L.M.G., Maia, A.S. and Souza, A.G. (2012). Commercial antioxidants and thermal stability evaluations. *Fuel, 97,* 638-643.

Shahidi, F. and Naczk, M. (1995). Nutritional and pharmacological effects of food phenolics. In: *Foodphenolics: Sources, chemistry, effects and applications (pp. 171–91).* Lancester, Pa.: Technomic Publishing Company, Inc.

Sivam, A.S., Sun-Waterhouse, D., Quek, S.Y. and Perera, C.O. (2010). Properties of bread dough with added fibre polysaccharides and phenolic antioxidants: A review. *Journal of Food Science, 75,* R163-R174.

Slavin J.L., Jacobs, D. and Marquart, L. (2000). Grain processing and nutrition. *Critical Reviews in Food Science and Nutrition, 40,* 309-326.

Song, W., Derito, C.M., Liu, M.K., He, C.J., Dong, M. and Liu, R.H. (2010). Cellular antixodiant activity of common vegetables. *Journal of Agricultural and Food Chemistry, 58,* 6621-6629.

Stoddart, M.J. (2011). Cell viability assays: Introduction. In: M.J. Stoddart (Ed.). *Mammalian Cell Viability: Methods and Protocls, Methods in Molecular Biology (pp. 1-6)*. Totowa: Humana Press Inc.

Sun-Waterhouse, D., Sivam, A.S., Cooney, J., Zhou, J., Perera, C.O. and Waterhouse, G.I.N. (2011). Effects of added fruit polyphenols and pectin on the properties of finished breads revealed by HPLC/LC-MS and Size-Exclusion HPLC. *Food Research International, 44,* 3047-3056.

Swieca, M., Gawlik-Dziki, U., Dziki, D., Baraniak, B. and Czyz, J. (2013). The influence of protein–flavonoid interactions on protein digestibility in vitro and the antioxidant quality of breads enriched with onion skin. *Food Chemistry, 141,* 451-458.

Tu, T., Giblin, D. and Gross, M.L. (2011). A structural determinant of chemical reactivity and potential health effects of quinines from natural products. *Chemistry Research in Toxicology. 24,* 1527-1539.

Utsumi, H. and Yamada, K. (2003). In vivo electron spin resonance-computed tomography/nitroxyl probe technique for non-invasive analysis of oxidative injuries. *Archives of Biochemistry and Biophysics, 416,* 1-8.

Vaidyanathan, S. and Bunzel, M. (2012). Development and application of a methodology to determine free ferulic acid and ferulic acid ester-linked to different types of carbohydrates in cereal products. *Cereal Chemistry, 89,* 247-254.

Vignais, P.V. and Vignais, P.M. (2010). *Discovering Life, Manufacturing Life: How the experimental method shaped life sciences*. New York: Springer.

Vitaglione, P., Napolitano, A. and Fogliano, V. (2008). Cereal dietary fibre: A natural functional ingredient to deliver phenolic compounds into the gut. *Trends in Food Science and Technology, 19,* 451-463.

Whent, M., Huang, H., Xie, Z., Lutterodt, H., Yu, L., Fuerst, E.P., Morris, C.F., Yu, L. and Luthria, D. (2012). Phytochemical composition, anti-inflammatory, and antiproliferative Activity of whole wheat flour. *Journal of Agricultural and Food Chemistry, 60,* 2129-2135.

Wilms, L.C., Kleinjans, J.C., Moonen, E.J. and Briede, J.J. (2008). Discriminative protection against hydroxyl and superoxide anion radicals by quercetin in human leucocytes in vitro. *Toxicology In Vitro,22*, 301–307.

Wolfe, K.L. and Liu, R.H. (2007). Cellular antioxidant activity (CAA) assay for assessing antioxidant, foods, and dietary supplements. *Journal of Agricultural and Food Chemistry, 55,* 8896-8907.

Wolfe, K.L., Kang, X.M., He, X.J., Dong, M., Zhang, Q.Y. and Liu, R.H. (2008). Cellular antioxidant activity of common fruits. *Journal of Agricultural and Food Chemistry, 56,* 8418-8426.

Yen, G.C., Duh, P.D., Tsai, H.L. and Huang, S.L. (2003). Pro-oxidative properties of flavonoids in human lymphocytes. *Bioscience, Biotechnology, and Biochemistry, 67,* 1215–22.

Yin, L., Wei, Y., Wang, Y., Xu, Y. and Yang, Y. (2013). Long term and standard incubations of WST-1 reagent reflect the same inhibitory trend of cell viability in rat airway smooth muscle cells. *International Journal of Medical Sciences, 10,* 68-72.

Young, J.C. and Abdel-Aal, E.M. (2010). Anthocyanins. In: P.R. Shewry and J.L. Ward (Eds). *HEALTHGRAIN methods: Analysis of Bioactive components in small grain cereals*. AACC International Press: St. Paul, Minnesota.

Yu, L., Nanguet, A-L. and Beta, T. (2013). Comparison of antioxidant properties of refined and whole wheat flour and bread. *Antioxidants, 2, 370-383*.

Yuan, X.P., Wang, J., Yao, H.Y. and Chen, F. (2005). Free radical scavenging capacity and inhibitory activity on rat erythrocyte hemolysis of feruloyl oligosaccharides from wheat bran insoluble dietary fiber. *Swiss Society of Food Science and Technology, 38,* 877-883.

Zhang, Y., Wang, L., Yao, Y., Yan, J. and He, Z. (2012). Phenolic acid profiles of Chinese wheat cultivars. *Journal of Cereal Science, 56,* 629-235.

Zieliski, H. and Kozowska, H. (2000). Antioxidant activity and total phenolics in selected cereal grains and their different morphological fractions. *Journal of Agricultural and Food Chemistry, 48,* 2008-2016.

APPENDIX

Appendix A. ANOVA table of FPC (FAE)

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	2	552.183	276.091	18.94	<.0001
process	6	61485.8	10247.6	703.01	<.0001
type*process	12	74.6066	6.21722	0.43	0.9348

Appendix B. ANOVA table of FPC (GAE)

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	2	265.605	132.803	18.94	<.0001
process	6	29575.3	4929.21	703.01	<.0001
type*process	12	35.8865	2.99054	0.43	0.9348

Appendix C. ANOVA table of DPPH values for free phenolic extracts

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	2	2651.64	1325.82	26.37	<.0001
process	6	234127	39021.2	776.09	<.0001
type*process	12	1716.06	143.005	2.84	0.0174

Appendix D. ANOVA table of ABTS values for free phenolic extracts

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	2	6696.91	3348.46	19.93	<.0001
process	6	1136154	189359	1126.96	<.0001
type*process	12	635.314	52.943	0.32	0.9784

Appendix E. ANOVA table of BPC (FAE)

Source	DF	Type I SS	Mean Square	F Value	Pr > F

type	2	101.394	50.697	2.33	0.1218
process	6	9221.99	1537	70.69	<.0001
type*process	12	6.415	0.53458	0.02	1

Appendix F. ANOVA table of BPC (GAE)

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	2	48.7714	24.3857	2.33	0.1218
process	6	4435.86	739.31	70.69	<.0001
type*process	12	3.08568	0.25714	0.02	1

Appendix G. ANOVA table of DPPH values for bound phenolic extracts

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	2	7886.63	3943.32	22.53	<.0001
process	6	150128	25021.3	142.97	<.0001
type*process	12	498.131	41.5109	0.24	0.9933

Appendix H. ANOVA table of ABTS values for bound phenolic extracts

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	2	49933.7	24966.9	24.49	<.0001
process	6	774430	129072	126.58	<.0001
type*process	12	7101.17	591.764	0.58	0.8338

Appendix I. ANOVA table of TAC (direct measurement)

Source	DF	Type I SS	Mean Square	F Value	Pr > F

type	2	287.33	143.665	10945.3	<.0001
process	6	70.7846	11.7974	898.8	<.0001
type*process	12	31.4003	2.61669	199.36	<.0001

Appendix J. ANOVA table of TAC (pH differential method)

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	1	84.9146	84.9146	1429.17	<.0001
process	6	93.5733	15.5955	262.48	<.0001
type*process	6	6.87294	1.14549	19.28	<.0001

Appendix K. ANOVA table of DPPH values for anthocyanin extracts

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	2	12952	6476.01	18.71	<.0001
process	6	191093	31848.8	92	<.0001
type*process	12	3242.56	270.214	0.78	0.6642

Appendix L. ANOVA table of ABTS values for anthocyanin extracts

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	2	46700.1	23350.1	101.67	<.0001
process	6	111308	18551.3	80.77	<.0001
type*process	12	12169	1014.09	4.42	0.0015

Appendix M. ANOVA table of soluble-free phenolic acids (HPLC)

***p*-Hydroxybenzoic acid**

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	3	27.0213	9.00711	2.98	0.0966
process	2	297.902	148.951	49.23	<.0001
type*process	2	9.0211	4.51055	1.49	0.2816

Vanillic acid

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	2	4.28743	2.14372	9.65	0.0058
process	3	6.84632	2.28211	10.28	0.0029
type*process	1	7.43945	7.43945	33.5	0.0003

***p*-Coumaric acid**

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	2	0.47884	0.23942	36.51	0.001
process	2	0.24543	0.12272	18.72	0.0048
type*process	0	0	.	.	.

Ferulic acid

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	2	21.8859	10.943	12.4	0.0003
process	6	736.058	122.676	139.01	<.0001
type*process	12	8.17368	0.68114	0.77	0.6718

Appendix N. ANOVA table of insoluble-bound phenolic acids (HPLC)

Protocatechuic acid

Source	DF	Type I SS	Mean Square	F Value	Pr > F
type	2	64.4271	32.2135	153.48	<.0001
process	4	1238.58	309.645	1475.29	<.0001
type*process	8	31.6896	3.9612	18.87	<.0001
***p*-Hydroxybenzoic acid**					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
type	2	56.268	28.134	50.21	<.0001
process	6	1848.72	308.12	549.94	<.0001
type*process	12	253.209	21.1008	37.66	<.0001
Vanillic acid					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
type	2	291.597	145.798	344.03	<.0001
process	6	521.577	86.9296	205.12	<.0001
type*process	12	149.944	12.4953	29.48	<.0001
Caffeic acid					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
type	2	20.0778	10.0389	303.84	<.0001
process	5	10.8113	2.16225	65.44	<.0001
type*process	10	3.39328	0.33933	10.27	<.0001
Syringic acid					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
type	2	23.2843	11.6422	111.92	<.0001
process	4	10.3617	2.59042	24.9	<.0001
type*process	8	12.2488	1.5311	14.72	<.0001
***p*-Coumaric acid**					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
type	2	494.826	247.413	302.65	<.0001
process	6	196.245	32.7076	40.01	<.0001
type*process	12	28.5263	2.37719	2.91	0.0156
Ferulic acid					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
type	2	33489.5	16744.7	37.1	<.0001
process	6	162371	27061.8	59.96	<.0001
type*process	12	4947.8	412.317	0.91	0.5502
Sinapic acid					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
type	2	3561.19	1780.6	269.89	<.0001
process	6	1582.65	263.774	39.98	<.0001
type*process	12	207.294	17.2745	2.62	0.0258

Appendix O. ANOVA table for cytotoxicity of digested bread extracts at different concentrations in different cell lines (MTT assay)

HT-29 cell line					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
Conc.	4	1.28919	0.3223	197.33	<.0001
breadtype	2	0.03661	0.01831	11.21	<.0001
conc*breadtype	8	0.11179	0.01397	8.56	<.0001
Caco-2 cell line					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
Conc.	4	0.06576	0.01644	133.83	<.0001
breadtype	2	0.0002	1E-04	0.81	0.4485
conc*breadtype	8	0.00037	4.6E-05	0.38	0.9291
FHs cell line					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
Conc.	4	0.076	0.019	483.93	<.0001
breadtype	2	0.00045	0.00022	5.73	0.0052
conc*breadtype	8	0.00066	8.3E-05	2.11	0.0477

Appendix P. ANOVA table for cytotoxicity of AAPH and H_2O_2 at various concentrations in different cell lines (MTT assay)

HT-29 cell line					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
Conc.	7	0.30367	0.04338	3714.32	<.0001
Stressor (AAPH & H_2O_2)	1	0.03031	0.03031	2594.72	<.0001
conc*stressor	4	0.0096	0.0024	205.58	<.0001
Caco-2 cell line					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
Conc.	7	0.22585	0.03226	3153.59	<.0001
Stressor (AAPH & H_2O_2)	1	0.00086	0.00086	83.63	<.0001
conc*stressor	4	0.00139	0.00035	34.04	<.0001

Appendix Q. ANOVA table for cytoprotectivity of digested bread extracts at different concentrations against AAPH induced oxidation in different cell lines

HT-29 cell line					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
conc	4	0.43145	0.10786	305.14	<.0001
breadtype	2	0.00723	0.00362	10.23	0.0001
conc*breadtype	8	0.00266	0.00033	0.94	0.4899
Caco-2 cell line					
	DF	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
conc	4	0.00237	0.00059	21.72	<.0001
breadtype	2	0.00133	0.00067	24.43	<.0001
conc*breadtype	8	0.00039	4.8E-05	1.77	0.1006
FHs cell line					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
conc	4	0.01393	0.00348	213.59	<.0001
breadtype	2	0.00071	0.00036	21.84	<.0001
conc*breadtype	8	0.00122	0.00015	9.39	<.0001

Appendix R. ANOVA table for cytoprotectivity of digested bread extracts at different concentrations against H_2O_2 induced oxidation in different cell lines

HT-29 cell line					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
conc	4	1.28919	0.3223	197.33	<.0001
breadtype	2	0.03661	0.01831	11.21	<.0001
conc*breadtype	8	0.11179	0.01397	8.56	<.0001
Caco-2 cell line					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
conc	4	0.06576	0.01644	133.83	<.0001
breadtype	2	0.0002	1E-04	0.81	0.4485
conc*breadtype	8	0.00037	4.6E-05	0.38	0.9291
FHs cell line					
Source	**DF**	**Type I SS**	**Mean Square**	**F Value**	**Pr > F**
conc	4	0.076	0.019	483.93	<.0001
breadtype	2	0.00045	0.00022	5.73	0.0052
conc*breadtype	8	0.00066	8.3E-05	2.11	0.0477