Antioxidant Activity of Commercial Wild Rice and Characterization of Phenolic Compounds by HPLC-DAD-ESI-MS/MS

By

Yang Qiu

submitted to the University of Manitoba

in partial fulfilment of the requirement of the degree of

MASTER OF SCIENCE

Department of Food Science

University of Manitoba

Winnipeg

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Antioxidant Activity of Commercial Wild Rice and Characterization of Phenolic Compunds by HPLC-DAD-ESI-MS/MS

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

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ABSTRACT

Wild rice (*Zizania*) as the only cereal native to North America played an important role in Americans' life in history. Due to the nutritional value and unique taste, it is currently used in a wide range of gourmet food products. In order to signify the potential health benefits to be derived from consumption of this cereal grain, the antioxidant capacities of eleven commercial wild rice samples (raw, mixed, and processed) were investigated in this study and their phenolic profiles were explored using HPLC-DAD-ESI-MS/MS techniques. Two solvent systems, aqueous methanol and acidic acetone, were involved in the extraction of phenolic compounds. The total phenolic contents (TPC) and antioxidant activities (AOA) of these two extracts were determined by Folin-Ciocalteau assay, DPPH photometric method and oxygen radical absorbance capacity (ORAC) assay. The results showed that the methanol extracts and acetone extracts contained comparable levels of TPC. The higher DPPH radical scavenging ability was found in acetone extracts, whilst methanol extracts showed greater ORAC values. Wild rice, compared with white rice (control sample), was more effective as an antioxidant. Significant differences ($p<0.05$) in antioxidant activities were detected among raw, mixed and processed samples. Several phenolic acids and their derivatives were detected in alkaline hydrolysates of methanol extracts and the remaining residues. Ferulic acid and sinapic acid were found in the most quantities, mainly occurring in insoluble bound form. Other monomeric phenolic acids detected in wild rice consisted of *p*-coumaric, vanillic, syringic, and *p*-hydroxybenzoic acids, along with two phenolic aldehydes (vanillin and *p*-hydroxybenzaldehyde). Phenolic acid dehydrodimers are cell wall bound and only appeared in the insoluble fractions featured by diferulic acids (DFA) and disinapic acids (DSA). The isomers of DFA included 8-8', 5-5', 8-O-4', and 8-5' (benzofuran form) coupled dimers, with 8-O-4' as the predominant form. DSA only

appeared as 8-8' coupled with the noncyclic isomers in most quantities. The antioxidants identified in acetone extracts were flavonoid glycosides (di-glucosyl apigenin, glucosyl-arabinosyl apigenin, and di-arabinosyl apigenin) and flavan-3-ols (catechin, epicatechin and oligomeric procyanidin). The above identified phenolic acids and flavonoids made significant contribution to the antioxidant activity of wild rice.

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LIST OF ABBREVIATIONS

3M	Three mixed wild rice
AAPH	2,2'-azobis (2-amino-propane) dihydrochloride
AB	A black wild rice
AOA	Antioxidant activity
AUC	Area under the curve
BB	B black wild rice
CL	Canadian lake wild rice
CS	C scarified wild rice
DAD	Diode array detection
DPPH	2,2'-diphenyl-1 picryl hydrazyl
DFA	Diferulic acid
DSA	Disinapic acid
EIC	Extracted ion chromatogram
ESI	Electrospray ionization
FAE	Ferulic acid equivalent
FL	Fluorescein
GAE	Gallic acid equivalent
HAT	Hydrogen atom transfer
HH	Hand harvested wild rice
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatograph – mass spectrometry
LS	Large sized wild rice
M	Manomin wild rice
MC	Minnesota cultivated wild rice
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
ORAC	Oxygen radical absorbance capacity
Q-TOF	Quadrupole time-of-flight
SET	Signal electron transfer
SS	Small sized wild rice
TIC	Total ion chromatogram
TE	Trolox equivalent
TMS	Trimethylsilyl
TPC	Total phenolic content
WR	White rice

CHAPTER 1: Literature Review

1.1. Introduction

Wild rice (common name Canadian rice, Indian rice or water oats) is an aquatic grass contained in the genus *Zizaniae*. Along with white, black and red rice (*Oryza*), it belongs to the cereal tribe *Oryzoidae* (Christou, 1994). As the only cereal indigenous to North America, it grows mainly in the northern United States and southern Canada. Historically, wild rice played an important role in Native Americans' life and was consumed as a staple food (Steeves, 1952), whereas in modern times, it is widely used in gourmet food products such as soups, salads, desserts, and meat dishes due to its unique toasted flavour and texture (Morton, Snyder, & Oelke, 1980). On the market, wild rice grains are generally sold in raw form. In addition to pure raw products, processed wild rice, namely quick-cook wild rice and blending wild rice which is mixed with one or more types of white rice are also commercially available.

The nutritional value of wild rice is equal to or perhaps even higher than other cereals (Anderson, 1976). It is high in carbohydrate and protein, but low in fat. Starch as the predominant compound makes up 74% of the dry matter of the grain (Hoover, Sailaja, & Sosulski, 1996; Lorenz, 1981a; Sosulski, 1993). The protein contents in wild rice ranges from 12.88% to 13.41%, with a low fat content less than 1% (Oelke & Boedicker, 2000; Terrell & Wiser, 1975). Other compounds such as dietary fibre and ash represent 6.8 and 1.8% of wild rice kernel weight, respectively (Anderson, 1976).

Wild rice kernel comprises a pericarp, aleurone, endosperm, and germ. Thus, it was recognized as a whole grain by the U.S. Food and Drug Administration (FDA) in 2006.

Consumption of whole grains has been associated with the reduction in incidences of cancer, chronic diseases, diabetes, and obesity (Slavin, 2004). It has been universally accepted that health benefits of whole grain are partially attributed to their unique phytochemical composition which function as natural antioxidants (Liu, 2007). The phytochemical profiles and antioxidant properties of cereals have been intensively studied in recent years, but mostly focused on barley, wheat and oats. Only limited literature reported the antioxidant activity of wild rice and focused mainly on the role in lipid peroxidation. Methanolic extract of wild rice grain or wild rice hull had capacity to retard lipid oxidation and enhance the stability of ground beef (Asamarai, et al 1996; Wu, 1994). Crude or cooked wild rice grain, when incorporated into meat products, showed good inhibitory activity in lipid oxidation implying them as effective antioxidants (Johnson et al, 1996; Katsanidis et al, 1997; Minerich et al, 1991; Rivera et al, 1996). However, so far the specific antioxidant constituents in wild rice grains have not been well studied with the exception of phytic acids (Becker & Lorenz, 1981; Wu, 1994).

Phenolic compounds as the secondary metabolites are widely distributed in plant kingdom. Plant based foods such as fruits, vegetables, and cereal grains contain a wide range of phenolic compounds which have been demonstrated as antioxidant constituents. The most common phenolics encountered in cereals are flavonoids and phenolic acids. The phenolic profiles of rice have been intensively studied. However, most research were focused on white, red and black rice (*Oryza* sp.) instead of wild rice (*Zizania* sp.), due to the less commercial importance of the latter. In recent years, Bunzel et al (2001) reported that like other cereals, diferulates were found in wild rice dietary fibre as structural components, along with disinapates. Several monomeric phenolic acids including ferulic acid, sinapic acid and *p*-coumaric acid disinapates were also detected in wild rice cell wall (Bunzel et al, 2002). However, phenolic acid

profiles in wild rice whole grain have not been reported. In addition to phenolic acids, flavonoid compounds have also not been characterized. The primary aim of this study was to identify major antioxidant compounds in commercial wild rice whole grain. A secondary objective of the investigation was to evaluate and compare the antioxidant properties of raw, mixed and processed wild rice samples.

1.2. Wild rice

1.2.1. The crop

1.2.1.1 Species and geological distribution

Wild rice (common name Canadian rice, Indian rice or water oats) is a group of aquatic grasses sharing the genus *Zizania*. It grows annually in shallow lakes and marshes. Although the genus *Zizania* is composed of wild rice, it is distantly related to the genus *Oryza* to which white rice belongs. They both belong to the cereal tribe *Oryzoidae*. Worldwide, there are four species of wild rice that have been identified.

Zizania palustris L. (Northern wild rice) grows in the region of south-eastern Manitoba and Ontario, but more abundantly in the North Central States of the U.S. Several years back, it was introduced into New Brunswick and Nova Scotia (Morton, Snyder, & Oelke, 1980).

Zizania aquatica L. (Southern wild rice) grows in the muddy streams from Quebec to southern Ontario and extending south to Louisiana and Florida (Chambliss, 1940). It is widely

distributed throughout the eastern half of the United States and is more plentiful in central and northern Minnesota (Moyle, 1944).

Zizania texana A.S. *Hitchc.* (Texas wild rice) originally grew in the headwaters of the river in Spring Lake, San Marcos, hays County, south-central Texas. However, due to the environmental pollution, it is in danger of extinction and currently found in the San Marcos River, within the city limits of San Marcos (Terrell, Emery, & Beaty, 1978).

Zizania latifolia Turcz. (Manchurian wild rice) is an Asian species, especially native to China and Japan (Okua 1978). In China, it is predominantly grown in the region surrounding Tai Lake, in the provinces of Jiangsu and Zhejiang (Guo et al, 2007).

Among these four species, *Zizania palustris* L. is of the most commercial importance and harvested mostly as a cereal grain (Vennum, 1988).

1.2.1.2. Domestication and commercial production

Prior to the 19th centuries, wild rice was naturally growing in limited areas of Canada and the United States. However, due to overharvesting, natural stands of wild rice were seriously destroyed and diminished by 1951. To conserve the natural stands and ensure a sustainable harvest, numerous efforts have been made. In 1950 to 1952, wild rice seeds collected from natural stands were initially cultivated in a crop field. Despite good yields in the first few years following cultivation, the crop was devastated by plant diseases. Thus, it became necessary to domesticate a good variety with pest- and shattering-resistance. Successful domestication of wild rice was not accomplished until 1960. As reported by Morton et al (1980), the domesticated wild

rice has a yield 4 to 14 times higher than the natural stands, thus permitting tremendous expansion in wild rice production.

To date, three commercial production systems of wild rice have been established in both Canada and the United States including natural stands, managed natural stands and paddy-grown stands (Hayes, Stucker, & Wandrey, 1989). In the first system, wild rice is naturally grown in shallow water and harvested in traditional canoe-flail way. Managed natural stands, in comparison to natural stands, are harvested mechanically instead of by hand. Paddy wild rice is cultivated in a crop field by managing water levels, improving or maintaining outlets, and assessing habitat.

Although, natural wild rice stands continue to be harvested, the production has steadily declined over years. Today, most commercial production of wild rice grain is derived from paddy-grown stands. According to the production statistics released by Minnesota Department of Agriculture and USDA-NASS, cultivated (paddy-grown) wild rice accounted for the greatest percentage of the global production (more than 95 %) (Hayes, Stucker, & Wandrey, 1989; Oelke & Boedicker, 2000).

1.2.1.3. Harvesting and processing

As a staple food for Native Americans, wild rice (*Z. palustris* L. or *Z. aquatica* L) has been harvested by Objiway, Manomini, and Cree tribes for centuries (Steeves, 1952). The traditional harvesting method was using a canoe and two wooden sticks, namely flails (Moyle, 1944). Usually, one harvester propels a canoe to go through the wild rice bed, while the other

harvester uses a flail to bend the stalk over the edge of the canoe, and knocks off the ripe grains into the boat with the other flail. To date, most natural wild rice stands are still harvested using this method. However, paddy stands are usually mechanically harvested by air boats. Since wild rice grains ripen over a period varying from 10 days to 2 weeks (Steeves, 1952) and the mature grains are easy to fall back into the water, stands in a rice bed must be harvested several times, usually at 2-day intervals (Moyle, 1944).

Freshly harvested wild rice contains a high moisture content ranging from 35% to 45% (Oelke & Boedicker, 2000). Thus, they must undergo a series of postharvest processing prior to long term storage. Prior to civilization, the grains were firstly dried in the sun or on a platform over a fire and then heated and stirred in a large iron kettle until most kernels were popped with husks being brown and brittle. The removal of hulls from hulled grains was achieved by dancing on or beating the grains with poles, followed by winnowing and fanning.

In more recent times, the processing is usually accomplished in a plant with a procedure involving fermentation, curing, parching (or heating) and hulling. Prior to fermentation, the grains are usually fractionated into three fractions consisting of mature kernels (heavy fraction), immature kernels (mediate fraction), and empty hulls (light fraction), respectively. After discarding the empty hulls, mature kernels in some plants are parched directly, bypassing the fermentation processing, whereas immature kernels must be fermented. Fermentation is a process where grains are stirred periodically in an open field with water added daily to keep the grain moist until the kernels take on a uniform characteristic blackish-brown color. It usually lasts for 4 to 7 days. This process not only imparts grain some special aroma but also cause deterioration of the tough hulls of wild rice seeds to facilitate hulling. Curing or drying is an

essential process which results in the reduction of the moisture content of the grain to about 7 % (Oelke & Boedicker, 2000). The unique toasted flavour of wild rice is also developed from this step. After hulling, the kernels still contain an outer seed coat or bran. Part of the bran is optionally scarified to reduce the cooking time. However, scarification is not necessary, its use being dependent on the raw products desired.

1.2.2. The grain

1.2.2.1. Kernel structure and generally chemical composition

When wild rice is harvested, the hulls and caryopsis appear intact as a unit. After hulling, the kernels of mature grain vary from 0.3 to 0.6 inches in length and from 0.06 to 0.18 inches in diameter (Oelke & Boedicker, 2000).

Unlike refined white rice, wild rice contains all parts of a grain including embryo, endosperm, and bran from inside to outside. Thus, it was listed as a whole grain along with barley, oat and wheat by the United States Food and Drug Administration in 2006.

The embryo (or germ) makes up the inner portion of the wild rice kernel representing approximately 5 % of the caryopsis weight (Albrecht, Oelke, & Brenner, 1979). Only a small amount of proteins and trace minerals are located in this fraction.

The endosperm of wild rice occupies the greatest percentage (around 90 %) of the caryopsis weight (Albrecht, Oelke, & Brenner, 1979). It provides a majority of starch and also

contains storage proteins along with cell wall polymers. Only small amounts of vitamins, minerals, and dietary fibre are located in this fraction.

The bran of wild rice comprises two layers, namely the pericarp (outer bran) and the aleurone (inner bran), with the former representing about 5 % of the kernel weight (Albrecht, Oelke, & Brenner, 1979). The predominant compounds in bran are insoluble dietary fibre and B-vitamins and a small amount of proteins.

1.2.2.2. Nutritional composition

The nutritional value of wild rice appears to equal or perhaps surpass other cereals (Anderson, 1976). It is high in carbohydrate and protein, but low in fat (Anderson, 1976; Capen & Leclerc, 1948; Kennedy, 1924; Watts & Dronzek, 1981).

Carbohydrate takes the most percentage ranging from 77 % to 82% of the dry weight of wild rice grains (Watts & Dronzek, 1981). Among the carbohydrates, starch is present in significant quantities with sugar and dietary fibre to a much lesser degree. The average starch content in wild rice makes up about 74 % of the dry matter of the grain (Anderson, 1976), while sugar and dietary fibre only represent 6.8 % and 1.7 % of the kernel weight, respectively (Hoover, Sailaja, & Sosulski, 1996). The amylose content in wild rice starch reported by Lorenz (1981) was only about 2 % of the grain weight, whereas in more recent research it was reported as 29 % (Hoover, Sailaja, & Sosulski, 1996). The physico-chemical properties of wild rice starch are characterized by high water-binding capacity, enzyme susceptibility, and swelling ability

(Lorenz, 1981a). However, the solubility of wild rice starch is lower than that of wheat starch (Lorenz, 1981a).

The protein content in wild rice grain ranges from 13.8 % to 17.1 % (dry basis) (Oelke, 1976; Wang et al, 1978; Watts & Dronzek, 1981). The amino acid composition of wild rice protein was further determined by Oelke (1976), Zhai et al (1994; 2001). It was found that wild rice proteins comprised nine essential amino acids, among which lysine and methionine were present in the most abundance. The contents of nonessential amino acids such as alanine, arginine, and aspartic were almost two times higher than found in wheat (Oelke, 1976).

The fat content in wild rice is less than 1 % (Anderson, 1976). Lund (1977) studied the fatty acid composition of wild rice and found that linolenic acid in wild rice comprised a much higher proportion of the fat than in wheat, or oats. The high level of linolenic acid in the fat makes wild rice healthier than other cereals.

The mineral composition of wild rice was comparable with oat, wheat, and corn (Anderson, 1976). In comparison with white rice or brown rice, wild rice was much richer in potassium, phosphorus, sodium, sulphur, and chlorine (Capen & Leclerc, 1948). Furthermore, wild rice is also an excellent source of B vitamins which include thiamine, riboflavin, and niacin (Capen & Leclerc, 1948; Oelke, 1976).

1.2.2.3. Phytochemical constituents and their functions

1.2.2.3.1. Function as antioxidants

Phytochemicals (from the Greek word *phyto*, meaning plant) are bioactive chemical compounds that are naturally occurring in plants. Most phytochemicals have antioxidant activity and may protect plant tissue from free radical damage.

The antioxidant activity of wild rice has been studied by a few workers who focused on its role in lipid oxidation. Earlier studies showed that incorporation of cooked wild rice into beef patties was capable of inhibiting lipid oxidation and retarding the development of meat rancidity (Minerich et al, 1991). Later, Johnson et al (1994), Rivera et al (1996), and Katsanidis et al (1997) reported that wild rice grain, when added into fresh-frozen, cooked and precooked ground beef patties or pork sausage, prolonged the storage of meat products, an indication that it may serve as a natural antioxidant additive in food products. The methanol and ethanol extracts of wild rice grain or wild rice hull also exhibited antioxidant effectiveness (Wu et al, 1994).

The specific antioxidant initially reported in wild rice grain was phytic acid (Becker & Lorenz 1981), the occurrence of which was later confirmed by Wu et al (1994). The mechanism of phytic acid acting as an antioxidant and a chelating agent was that it could react with Fe^{3+} to form a chelate, thus preventing Fe^{3+} from catalyzing the formation of hydroxyl radicals in the Fenton reaction and the Haber-Weiss cycle (Graf, 1983). Due to their antioxidant activity, phytic acids have been suggested as potential alternative antioxidants for use in the food industry to improve the shelf life of food products (Graf & Eaton, 1990). The phytic acid content in wild rice ranged from 2.1 % to 2.4 % by weight (Becker & Lorenz, 1981).

1.2.2.3.2. Function as flavour compounds and pigments

Phytochemicals not only serve as natural antioxidants, but also play an important role in the flavour and pigment system of wild rice. Wild rice has a unique toasted or nutty flavour. By applying gas chromatography coupled with mass spectrometry, Withycombe (1974) detected 112 volatile compounds contributing to the distinctive flavour system of wild rice including pyrazines, furans, and phenolic constituents. However, the flavour compounds identified in white rice, in comparison to wild rice, were characterized by the abundant occurrence of alkylbenzenes, aldehydes, and 2-alkanones (Bullard & Holguin, 1977).

In addition to the unique flavour, wild rice kernels also have a distinctive color. The immature kernels present in a greenish color, whereas more mature ones appear in a black-brown color. The pigments identified in wild rice seeds were anthocyanins, carotenoid, tannins, and chlorophylls (Withycombe, 1974). Anthocyanins were not only reported in wild rice seeds, but also in the leaf sheath and staminate florets (Gutek, 1981). Gutek (1981) found that cyanidin 3-glucoside constituted approximately 75% of total anthocyanin content with the rest (25%) present as cyanidin 3-rhamnoglucoside.

1.2.2.4. Potential health benefits

Epidemiological studies have shown that regular consumption of whole grains has a great potential for reducing the risks of various types of chronic diseases (Anderson et al, 2000; Jacobs et al, 1998; Liu et al, 1999), diabetes (Chatenoud et al, 1998; Liu et al, 2000; Meyer et al, 2000), some cancers (Jacobs et al, 1995 & 1998; Kasum et al, 2002; Nicodemus et al, 2001) and all-cause mortality (Jacobs et al, 1999 & 2001). These health benefits are probably associated with the following components: phenolic compounds, dietary fibre, and vitamins (Liu, 2007).

Phenolic compounds as the most important group of phytochemicals can inhibit lipid peroxidation by scavenging free radicals such as hydroxyl radicals and peroxyl radicals which result in the formation of low energy phenolic radicals whose energy is not sufficient to promote lipid oxidation (Decker, 1998). Consequently, dietary phytochemical components may actively contribute to the control of oxidative reactions in the body. Consumption of dietary fibre helps to lower blood cholesterol levels and normalize blood glucose and insulin levels (Marlett et al, 2002). It is also helpful for cardiovascular disease and type II diabetes when making dietary fibre as part of the daily diet (Marlett et al, 2002).

1.3. Phenolic compounds

1.3.1. General introduction

Phenolic compounds or phenolics are plant secondary metabolites providing plants with diverse functions ranging from structural ones to protection (Harborne, 1980). To date, approximately 8000 naturally occurring phenolic compounds have been identified in the plant kingdom, all of which in common are composed of an aromatic ring bearing one or more hydroxyl groups (Harborne, 1980). According to the structural complexity, phenolics can be simply divided into two groups, namely simple phenols and polyphenols (Harborne, 1980). The simple classes are referred to those only containing one phenol subunit such as phenolic acids, whereas polyphenols comprise two or more phenol subunits and include mainly flavonoids and proanthocyaninis (condensed tannins).

Most plant phenolics are water soluble. They are usually present as sugar conjugates and located within the plant cell in the central vacuole (Harborne, 1980). A minority of phenolics are

lipophilic. They are generally occurring in the cell cytoplasm or at the surface of plants in waxes and bud exudates (Harborne, 1980).

1.3.2. Phenolic acids

1.3.2.1. Monomer: classification and chemical structure

The term "phenolic acids" is generally used to designate phenols that only possess one carboxylic acid functionality (Robbins, 2003). When it comes to plant metabolites, they are referred to as a distinctive group of organic aids (Stalikas, 2007).

The basic chemical structures of natural phenolic acids are distinguished by the following two frameworks: hydroxylcinnamic and hydroxybenzoic structures (Figure 1.1). Aldehyde analogues such as vanillin, are also grouped within and referred to as phenolic acids.

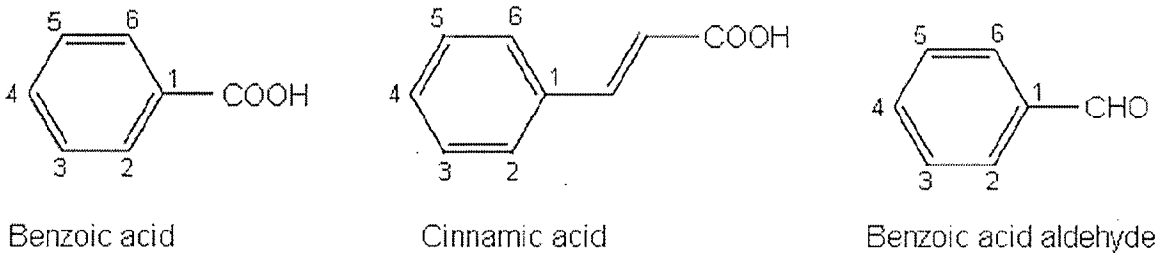


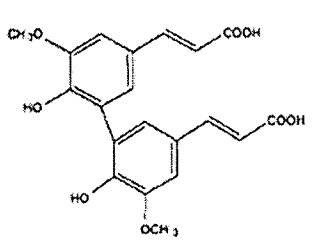
Figure 1.1. The basic frameworks of phenolic acids

The variation in the number and arrangement of the hydroxyl groups on the aromatic ring makes phenolic acids differ from individual to individual. In cereals, the most commonly encountered hydroxycinnamic acids are *p*-coumaric, caffeic, ferulic, and sinapic acids (Mattila & Kumpulainen, 2002). *p*-Hydroxybenzoic acid, vanillic acid, and protocatechuic acid are the most

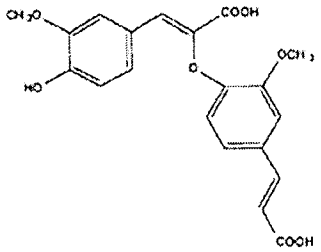
common hydroxybenzoic acids (Mattila & Kumpulainen, 2002).

1.3.2.2. Oligomeric phenolic acids in cereal dietary fibre

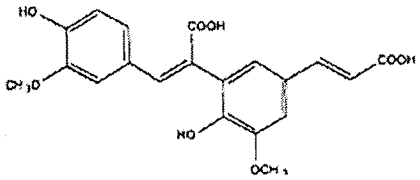
In cereals, phenolic acids are not only found as monomers, but are also present in the form of dimers, trimers, and even tetramers. Ferulic acid dehydrodimers (also referred to as diferulic acids or diferulates) are the most common cell wall esterified phenolic dimers which are particularly concentrated in cereal dietary fibre as structural constituents. To date, seven ferulic acid dehydrodimers have been identified (Bunzel et al, 2000, 2001, 2002, 2003, 2004, & 2005; Waldron et al, 1996) and their chemical structures are shown in Figure 1.2. These diferulates include 5,5'-coupled, 8-O-5'-coupled, 8,5'-coupled (noncyclic & cyclic forms), 8,8'-coupled (noncyclic & cyclic forms) and 4-O-5'-coupled diferulic acids. In addition to diferulic acids, disinapic acids were also detected in some cereal grains, especially in wild rice and spelt (Bunzel et al, 2003). However, unlike diferulic acids, they are only occurring as 8,8'-dehydrodimers with two isomers (noncyclic & cyclic forms).



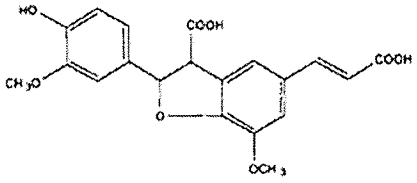
5,5'-diferulic acid



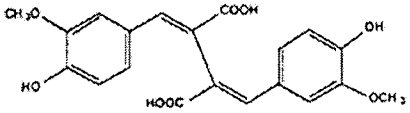
8-O-4'- diferulic acid



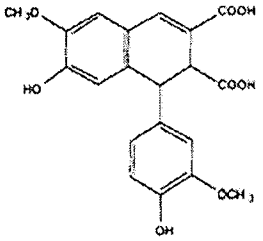
8,5'-diferulic acid (noncyclic form)



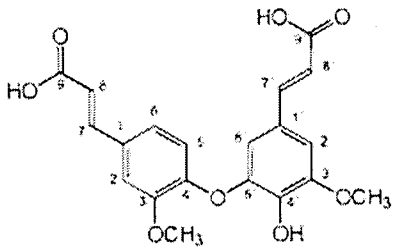
8,5'-diferulic acid (cyclic form)



8,8'-diferulic acid (noncyclic form)



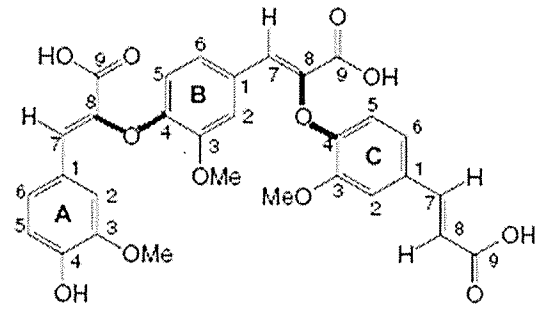
8,8'-diferulic acid (cyclic form)



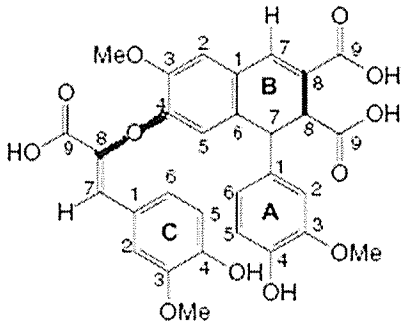
4-O-5'- diferulic acid

Figure 1.2. Chemical structures of diferulic acids (adapted from Waldron et al, 1996)

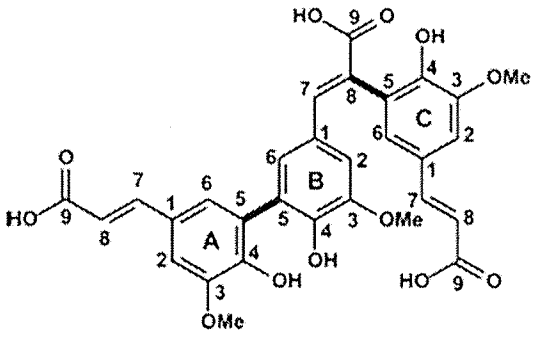
In addition to dehydrodimers, oligomeric ferulic acids with higher DP degrees (DP>2) are also detected in cereal grains. However, the structural identification has not been accomplished until recent years (Bunzel et al, 2005, 2006 & 2007). For dehydrotriferulates, the structures are identified as 8-O-4'/8-O-4'-coupled, 8-8'/8-O-4'-coupled, 8-5'(noncyclic form)/5-5'-coupled, and 8-8'(cyclic form)/5-5'-coupled dehydrotriferulic acid (Figure 1.3).



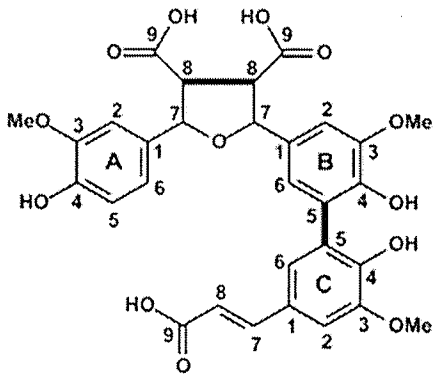
8-O-4'/8-O-4'-coupled



8-8'/8-O-4'-coupled



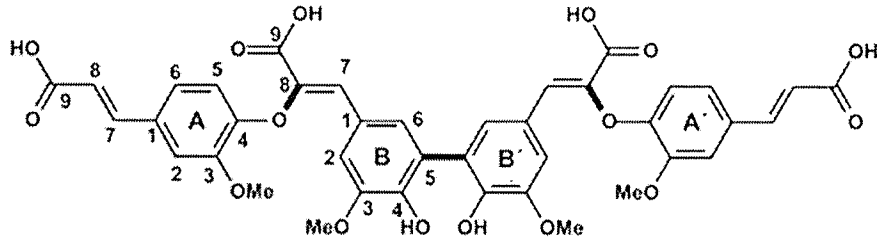
8-5'(noncyclic form)/5-5'- coupled



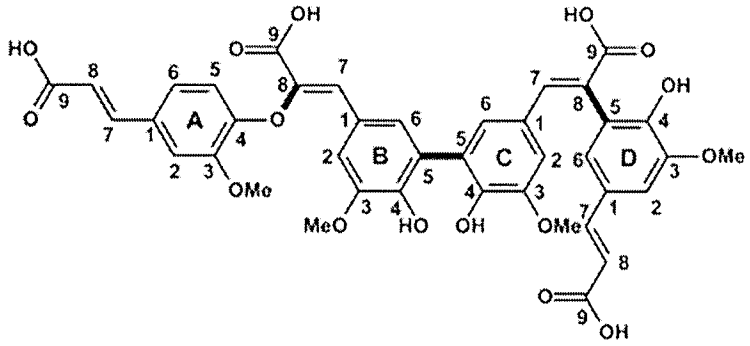
8-8'(cyclic form)/5-5'-coupled

Figure 1.3. Chemical structures of dehydrotriferulic acids (adapted from Bunzel et al, 2005)

Moreover, 4-O-8'/5-5'/8-O-4'-dehydrotetraferulic acid and 4-O-8'/5-5'/8-5'(noncyclic)-dehydrotetraferulic acid were first identified in 2006 (Figure 1.4).



4-O-8'/5-5'/8-O-4'-coupled



4-O-8'/5-5'/8-5'(noncyclic)-coupled

Figure 1.4. Chemical structures of dehydrotetraferulic acids (adapted from Bunzel et al, 2007)

1.3.2.2. Antioxidant properties

1.3.2.3.1. Mechanisms

The antioxidant properties of phenolic acids are attributed to the hydroxyl substituent on the aromatic ring (Rice-Evans, Miller, & Paganga, 1996). The Fenton reaction is one of the most powerful oxidation reactions involving a hydrogen peroxide and a ferrous iron catalyst.

$$H_2O_2 + Fe^{2+} \rightarrow \bullet OH + OH^- + Fe^{3+}$$

Under the catalyzation of ferrous iron, the hydrogen peroxide will generate a hydroxide ion and a hydroxyl radical. The latter, known as one of the highly aggressive oxygen species, can oxidize the organic molecules and result in cell damage.

During oxidative reactions, phenolic acids behave as hydrogen or electron donors. The hydroxyl group on the aromatic ring are able to break Fenton reaction to generate a less aggressive radical, thus repairing the activity of oxygen species.

$$PA\text{-}OH + R\bullet \rightarrow PA\text{-}O\bullet + RH$$

Two principle mechanisms involved in phenolic antioxidant properties: hydrogen atom transfer (HAT) and single electron transfer (SET) reactions (Dziedzic & Hudson, 1983; Shahidi, Janitha, & Wanasundara, 1992). When R• represents methoxyl or benoxyl radicals, the first mechanism (HAT) is involved; while for phenoxyl radicals, the second mechanism (SET) is the responsible one (Mayer et al, 2002).

1.3.2.3.2. Structure-activity relationship

The antioxidant activity of phenolic acids is dependent on the substitution patterns. Due to the variation in the number and arrangement of hydroxyl groups on the aromatic ring and

other factors, phenolic acids exhibited different levels of radical scavenging ability (Rice-Evans, Miller, & Paganga, 1996).

Generally, hydroxycinnamic acids are considered to be more effective than their hydroxybenzoic counterparts due to the presence of CH=CH-COOH group. Within the hydroxycinnamic group, the antioxidant activities decrease in the order of *p*-coumaric acid ferulic acid, caffeic acid, *m*- and *o*- coumaric acids (Rice-Evans, Miller, & Paganga, 1996). Among hydroxylated benzoates, gallic acid behaves more effectively than syringic acid, protocatechuic acid and *m*-hydroxylbenoic acid (Rice-Evans, Miller, & Paganga, 1996). No antioxidant activity is detected in *p*-hydroxylbenoic acid due to the *para* position of hydroxyl group (Rice-Evans, Miller, & Paganga, 1996). Furthermore, as reported by Ward et al (2001), diferulic acids have more antioxidant potency than monomeric ferulic acid. However, it is worth a mention that due to the variation in analytical procedures and methodologies, the antioxidant activities estimated for different phenolic acids may not be consistent in the literature.

1.3.2.4. Analytical methodology

Phenolic acids play an important role in food color and sensory qualities (Robbins, 2003). Their antioxidant properties make them further valuable for human health. Therefore, identification and structural characterization of phenolic acids occurring in plant-based foods and other food systems tend to be important. Robbins (2003) reviewed a plethora of methodologies and techniques used in analysis of phenolic acid.

As reviewed by Robbins (2003), high performance liquid chromatography coupled with diode array detection (HPLC-DAD) is the most common techniques applied in analysis of phenolic acids. Chromatographic separation of phenolic acids is usually carried out on a

reversed-phase (RP) C8 or C18 column (Robbins, 2003). For benzoic acid derivatives, the maximum UV absorption is in the range of 200 nm to 290 nm, with an exception of gentisic acid whose absorbance extends to 355 nm (Robbins, 2003). In comparison to hydroxybenzoic counterparts, hydroxycinnamates have a relatively broad UV absorption from 270 nm to 360 nm (Robbins, 2003). Thus, peak assignments for monomeric phenolic acids can be accomplished by comparing both retention times and UV spectra of analytes to commercial standards.

However, HPLC-DAD cannot accomplish the definitive identification of oligomeric phenolic acids, without the use of reliable MS spectrometric techniques. MS is one of the most effective methods for the identification, quantification, and structural determination of natural products from biological materials. The high sensitivity, specificity, and easy combination with chromatographic techniques have resulted in MS spectrometric techniques being widely used in the analysis of phenolic compounds.

Gas chromatography coupled with mass spectrometry (GC-MS) is the major technique for the analysis of small molecules (Prasain, Wang, & Barnes, 2004). Bunzel and his coworkers successfully used this technique to discover a series of oligomeric phenolic acids in cereal dietary fibre (Bunzel et al, 2001; 2002; 2003; 2004; 2005; 2006; 2007). The disadvantage of GC-MS is the need for derivatization of phenolic acids by using trimethylsilyl (TMS).

LC-MS is a valid alternative method for the identification of phenolic acids. The negative ion mode is usually employed due to the high sensitivity. The precursor ions $[M-H]^-$ determined in full scan along with retention times are also able to give unambiguous identification for monomeric phenolic acids. However, oligomeric phenolic acids have a range of isomers thus generating the same precursor ions. Therefore, the product ions obtained from MS/MS are used for the specific identification. The most common product ions observed in the MS/MS spectra

are $[M-15]^-$, $[M-30]^-$, and $[M-44]^-$ (Robbins, 2003). The fragment ion $[M-15]^-$ is caused by the loss of a methyl group via alpha-cleavage, while $[M-30]^-$ is formed by losing a molecule of formaldehyde and represents the cleavage of the methoxyl substituent of the phenyl ring (Robbins, 2003). The fragment $[M-44]^-$ involves the cleavage and rearrangement of a carboxyl group (Robbins, 2003). However, so far, there is no detailed information on the fragmentation pathway of phenolic acid oligomers.

1.3.3. Flavonoids

1.3.3.1. Aglycones: classification and chemical structure

Flavonoids are a large group of polyphenols. Although they are predominantly occurring as glycosides in plant cells, free aglycones (the unconjugated flavonoids without a sugar moiety) are also occasionally found in plant secondary metabolites.

The basic skeleton of aglycones is C_6-C_3-C_6, containing three rings labelled A, B, and C, respectively (Figure 1.5.). They are formed by a series of condensation reactions between hydroxycinnamic acids which contribute to the formation of B-ring and carbon atoms 2, 3 and 4 of C-ring, and malonyl residues which contribute to forming A-ring (Cuyckens & Claeys, 2004).

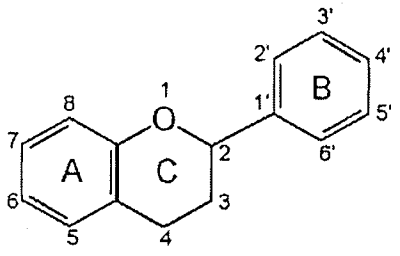


Figure 1.5. The basic chemical structure of aglycones

Based on the variation in the substitution patterns of the C-ring, aglycones are subdivided into several groups mainly comprising flavones, flavonols, isoflavones, flavanones, flavanonols, flavan-3-ols, and anthocyanidins, the chemical structures of which are depicted in Figure 1.6.

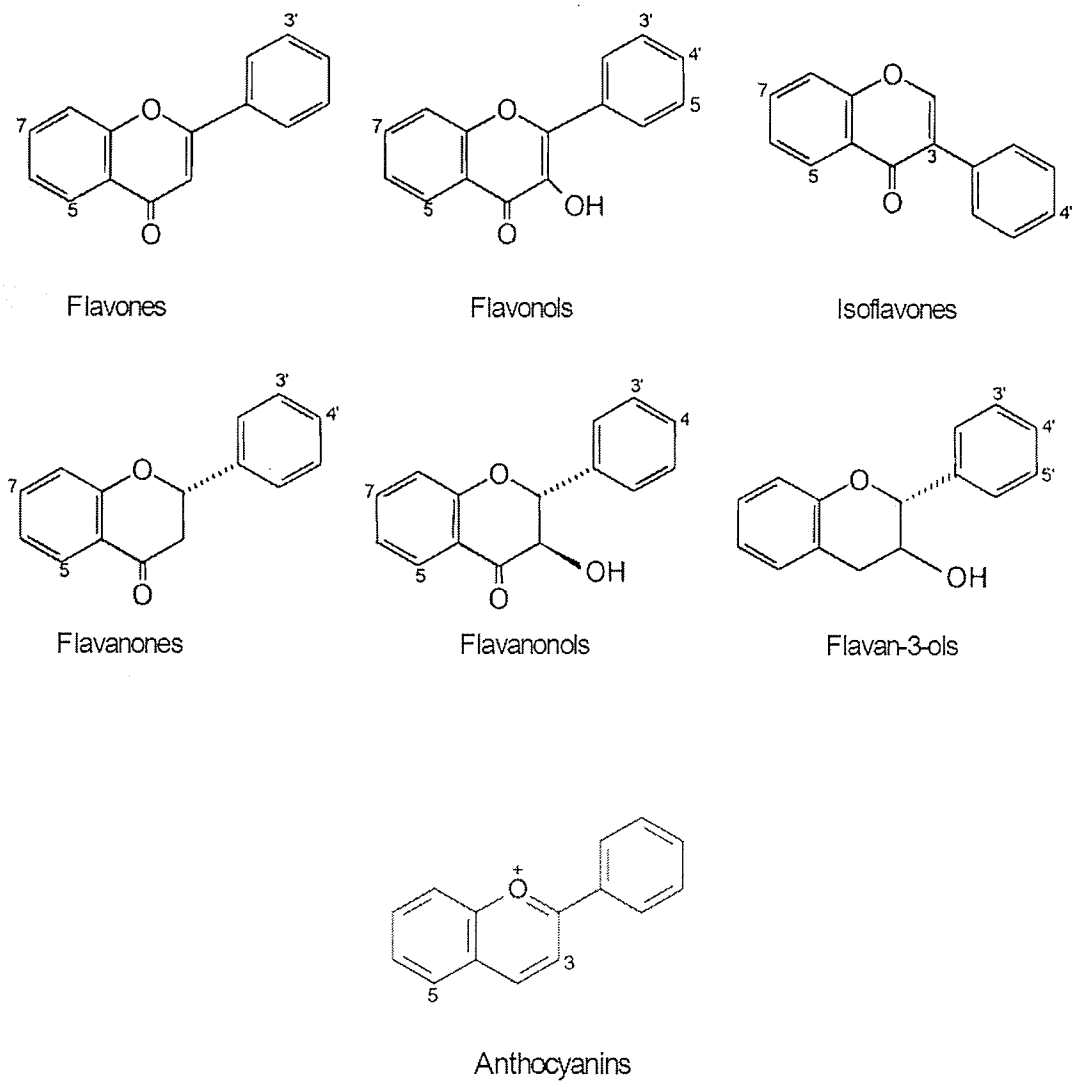


Figure 1.6. Structures of major classes of aglycones (adapted from Pietta, 2000)

The individual compounds within a class are varied in the substitution patterns of the A- and B-rings. The most frequent substitution positions are 3, 5, 7, 3', 4' and 5' with one or more hydroxyl groups which are usually methylated, acetylated, prenylated or sulphated (Cuyckens & Claeys, 2004). To date, about 400 flavone aglycones, 450 flavonol aglycones, 3300 isoflavone aglycones, 50 flavanone aglycones, and 19 anthocyanidins have been reported (Iwashina, 2000). Table 1.1 summarizes some of the representative aglycones in each class.

Table 1.1. Substitution of representative aglycones in each class

Class	3	5	7	3'	4'	5'
Flavones						
Chrysin		OH	OH			
Apigenin		OH	OH		OH	
Luteolin		OH	OH	OH	OH	
Flavonols						
Galangin	OH	OH	OH			
Kaempferol	OH	OH	OH		OH	
Fisetin	OH		OH	OH	OH	
Quercetin	OH	OH	OH	OH	OH	
Myricetin	OH	OH	OH	OH	OH	OH
Isoflavones						
Daidzein			OH		OH	
Daidzin			O-glc		OH	
Genistein		OH	OH		OH	
Genistin		OH	O-glc		OH	
Flavanones						
Naringenin		OH	OH		OH	
Hesperetin		OH	OH	OH	OCH_3	
Flavanonols						
Taxifolin	OH	OH	OH	OH	OH	
Flavan-3-ols						
(+)-Catechin	βOH	OH	OH	OH	OH	
(-)-Epicatechin	αOH	OH	OH	OH	OH	
(-)-Epigallocatechin	αOH	OH	OH	OH	OH	OH
Anthocyanidins or anthocyanins						
Apigenidin		OH	OH		OH	
Cyanidin	OH	OH	OH	OH	OH	
Cyanin	O-glc	OH	OH	OH	OH	

1.3.3.2. Flavonoid glycosides

Naturally occurring flavonoids are most often present as glycosides with the number of sugar residues varying from one to four. Sugar substitution on the aglycone skeleton may occur through hydroxyl groups with a formation of *O*-glycosides or directly bound to carbon atoms in ring A in the case of *C*-glycosides (Cuyckens & Claeys, 2004). In *O*-glycosides, the glycosylation usually takes place at 3 or 7 positions with hydroxyl groups to form an acid-labile hemiacetal O-C bond, whereas the *C*-glycosides generally have sugar groups substituted at 6 or 8 positions with a formation of an acid-resistant C-C bond (Cuyckens & Claeys, 2004). Generally, *O*-glycosides occur far more frequently than *C*-glycosides. For flavonol and flavanone aglycones, they are usually occurring as *O*-glycosides, whereas flavones are found in both forms. The most common sugar constituents conjugated with flavonoids are rhamnose, glucose, galactose and arabinose, whereas mannose and fructose are rare (Cuyckens & Claeys, 2004).

1.3.3.3. Antioxidant properties

1.3.3.3. 1. Mechanisms

Flavonoids are universally accepted as potent antioxidants. There are several probable mechanisms for antioxidant action of flavonoids as suggested by Halliwell and Gutteridge (1998), Pietta (2000), and Nijveldt et al (2001).

(a) Direct scavenging free radicals by hydroxyl groups

This mechanism is the same as the one discussed for phenolic acids. Hydroxyl groups substituted on flavonoids are able to donate a hydrogen atom or an electron to hydroxyl, peroxyl, or peroxynitrite free radicals with the generation of a more stable and less reactive flavonoid radical, thus resulting in directly radical scavenging.

(b) Suppressing free radicals by interaction with diverse enzyme systems

By reacting with enzymes systems, flavonoids are capable of suppressing reactive oxygen or nitric species. As reported by Pietta (2000), flavonoids have inhibitory effects on the enzymes responsible for superoxide anion production, such as xanthine oxidase and protein kinase C. Other enzymes involved in reactive oxygen species formation such as cyclooxygenase, lipoxygenase, and mitochondrial succinoxidase can be also suppressed by flavonoid compounds.

(c) Suppressing free radicals through chelating trace elements

Chelating trace elements is another pathway to suppress free radicals. Trace metals such as copper are potential enhancers of reactive oxygen species generation. With the presence of copper, low-density lipoprotein (LDL) can be easily oxidized into an aggressive radical.

$$LH \rightarrow L\bullet \rightarrow LOO\bullet$$

where LH represents a low-density lipoprotein. However, some specific flavonoids have iron-chelating ability, resulting in direct inhibition of lipid peroxidation. A typical example is quercetin.

1.3.3.3.2. Structure-activity relationship

The antioxidant activities of various flavonoids have been investigated by Rice-Evans and his co-workers (1996) and expressed as trolox equivalent antioxidant activity (TEAC). A structure-activity relationship is also established and suggested by several authors (Heim, Tagliaferro, & Bobilya, 2002; Rice-Evans, Miller, & Paganga, 1996). The major determinants for radical-scavenging capability are concluded as follows.

(a) Configuration and number of hydroxyl groups

Due to the radical scavenging ability as mentioned before, the substitution patterns of hydroxyl groups plays an important role in antioxidant activity of flavonoids. The arrangement of hydroxyl groups on B-ring is the most predominant determinant. A 3', 4'-catechol structure in B-ring efficiently enhances the lipid peroxidation inhibition. For instance, luteolin and kaempferol possess identical hydroxyl configuration, but the former shows a much higher level of antioxidant activity (2.1 mM TEAC) than the latter (1.34 mM TEAC), due to the presence of a catechol structure (Heim, Tagliaferro, & Bobilya, 2002; Rice-Evans, Miller, & Paganga, 1996). The number of hydroxyl groups on B-ring is also related with the radical scavenging ability. As reported by Cao et al (1997), among structurally homologous flavones and flavanones, peroxyl and hydroxyl scavenging increases linearly and curvilinearly respectively, based on the number of hydroxyl groups.

The hydroxyl groups on C-ring tend to be less important, but the presence of 3- hydroxyl substitution permits an enhancement in stability of flavonoid radicals thus elevating the overall antioxidant activity. The notable examples are flavonols vs. flavones and flavanols vs. flavanones. Flavonols (or flavanols) have been shown to have more antioxidant activity than the corresponding flavones (or flavanones) because of the occurrence of 3-OH. The significance of hydroxyl groups on the A-ring is not clear.

(b) 2, 3-Double bond and 4-oxo group

Although Burda and Oleszek (2001) found no correlation between 2, 3-double bond and antioxidant activity by DPPH method, most studies support that 2, 3-double bond conjugated with 4-oxo group are potential inhibitors of hydroxyl or peroxyl radicals. Flavanones and flavanonols, in comparison to flavones and flavonols, are generally considered as weak

antioxidants due to the lack of conjugation provided by the 2,3-double bond and the 4-oxo group (Pietta 2000).

(c) Glycosylation

Glycosylation diminishes the radical-scavenging capacity greatly. Generally, aglycones are more potent antioxidants than their corresponding glycosides. Moreover, *O*-methylation is also related with a decrease in antioxidant activity of flavonoids.

1.3.3.4. Analytical methodology

Due to the high prevalence of plant-derived foods in the human diet and their potential health benefits attributed to their antioxidant properties, flavonoids are of particular interest among plant polyphenols. The developments of rapid and reliable methods to analyze flavonoids tend to be important in food quality control.

HPLC coupled to multiple-wavelength or diode-array UV detection (DAD) and mass spectrometry (MS) has proved to be one of the most effective techniques in the analysis of flavnoids compounds. The UV spectra of all the flavonoids are characterized by two major absorption bands: band I in the range of 300 to 550 nm dependent on the substitution pattern and conjugation of the C-ring; band II in the range of 240–285 nm due to the A-ring (Mabry, Markham, & Thomas, 1970). The characteristic UV absorptions of selected flavonoids are summarized by Leth et al (1998) and listed in Table 1.2.

Table 1.2. The characteristic UV absorptions of selected flavonoid aglycones

Class	Aglycone	UV_{max}
Flavones	Apigenin	341
	Luteolin	351
	Isorhamnetin	372
Flavonols	Kaempferol	366
	Myricetin	375
	Quercetin	374
Flavanones	Nargenin	292
	Hesperitin	289
	Eriodictyol	288

Although HPLC-DAD enables distinction of the subclasses of flavonoids based on their characteristic UV absorption, it is difficult to identify the individual flavonoids without the help of standards and reference data. Modern mass spectrometric (MS) techniques are able to facilitate the identity and provide information on molecular mass and on structural characteristics for those unknown compounds.

Gas chromatography coupled with mass spectrometry (GC-MS) is not widely used in flavonoid analysis because of the limited volatility of flavonoid glycosides and the time consuming step of derivatization (Cuyckens & Claeys, 2004). Compared with GC-MS, LC-MS is more efficient and most commonly used in flavonoid analysis. A C8 or C18-RP column can give good separation of flavonoids. The molecular masses of separated flavonoids can be

obtained from a full mass scan. More detailed structural features can be obtained by tandem mass spectrometry including:

(a) the type of aglycone

(b) the number and type of sugar moiety (hexose and/or pentose)

(c) the interglycosidic linkages

(d) the other substituent and the attachment points to the aglycone (Cuyckens & Claeys, 2004).

However, the MS techniques are still not able to provide complete structural information. Other spectroscopic techniques such as NMR are needed to aid in the identification of the unknown flavonoid compounds. Therefore, the implementation of novel technologies to characterize polyphenols is still under way.

CHAPTER 2: Antioxidant properties of methanol extracts from commercial wild rice and analysis of soluble and insoluble phenolic acids

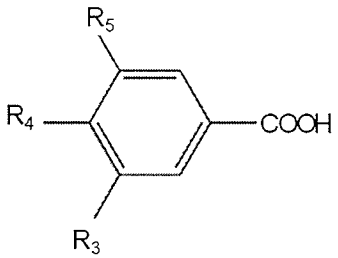
ABSTRACT

To investigate the antioxidant properties of commercial wild rice, identify and quantify soluble and insoluble phenolic acids in wild rice whole grain, the alkaline hydrolysates from crude methanol extracts (soluble fraction) and residues (insoluble fraction) were separately analyzed by reversed phase high performance liquid chromatography (HPLC) coupled with photodiode array detection (PAD) and quadrupole - time of flight (Q-TOF) mass spectrometry (LC-MS). The antioxidant activity of wild rice methanol extract was found to be up to 10 times greater than that of white rice (control sample) according to their DPPH radical scavenging activity and oxygen radical absorbance capacity (ORAC). Among wild rice samples, quick cooking wild rice (processed sample) and mixed wild rice displayed lower antioxidant capacity than raw samples. Total phenolic contents (TPC) in raw wild samples ranged from 419 to 588 mg gallic acid equivalent (GAE)/kg of rice. However, processed and mixed samples only consisted of 353 mg GAE/kg and 253 mg GAE/kg phenolic compounds, respectively. After alkaline hydrolysis, total phenolics released from methanol extracts and residue fractions ranged from 105 to 169 mg GAE/kg and 158 to 212 mg GAE/kg, respectively for raw samples. Ferulic acid was found as the most abundant phenolic acid (up to 355 mg/kg) in wild rice followed by sinapic acid. They both occurred mainly in the insoluble form. Other monomeric phenolic acids present in wild rice consisted of *p*-coumaric, vanillic, syringic, and *p*-OH-benzoic acids, along with two phenolic acid aldehydes (*p*-OH-benzaldehyde and vanillin). They were present in both

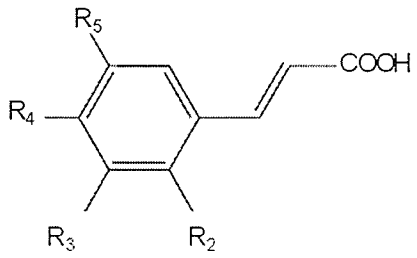
soluble and insoluble forms. Phenolic acid dehydrodimers are cell wall bound and only appeared in the insoluble fractions featured by diferulic acids (DiFA) and disinapic acids (DiSA). The chemical structures of DiFA included 8-8', 5-5', 8-O-4', and 8-5' (benzofuran form) coupled dimers, with 8-O-4' as the predominant form (up to 34 mg/kg). DiSA only appeared as 8-8' coupled products with the linear isomers in the most quantities (up to 19 mg/kg). The DPPH free radical scavenging activities of soluble and insoluble fractions suggest that the antioxidant activity of wild rice is partially attributed to its phenolic acid profile.

2.1. Introduction

Phenolic acids and their derivatives are universally distributed in the plant kingdom as the secondary metabolites. In recent years, there is an increasing interest in investigating phenolic acid profiles in plant derived foods including fruits, vegetables, and cereal grains due to their antioxidant activity and potential health benefits related to their protective effects on oxidative stress-induced diseases such as cancer, and cardiovascular diseases. The basic chemical structures of natural phenolic acids are distinguished by the following two frameworks: hydroxycinnamic and hydroxybenzoic acid derivatives (Figure 2.1).



Benzoic acids



Cinnamic acids

Derivatives	R_3	R_4	R_5
o-Hydroxybenoic	OH		
p-Hydroxybenoic		OH	
Protocatechuic	OH	OH	
Vanillic	OCH_3	OH	
Syringic	OCH_3	OH	OCH_3

Derivatives	R_2	R_3	R_4	R_5
o-Coumaric	OH			
p-Coumaric			OH	
Caffeic		OH	OH	
Ferulic		OCH_3	OH	
Sinapic		OCH_3	OH	OCH_3

Figure 2.1. Chemical structures of phenolic acids

The most commonly encountered hydroxycinnamic acids are *p*-coumaric, caffeic, ferulic, and sinapic acids. Hydroxybenzoic acids mainly consist of *p*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids. Along with these two major groups, aldehyde analogues are also grouped within and referred to as phenolic acids such as vanillin (Robbins, 2003). Cereal grains contain a wide range of phenolic acids with ferulic and *p*-coumaric acids in the most significant quantities (Herrmann, 1989; Mpofu, Sapirstein, & Beta, 2006). High concentrations of these compounds are found in the outer layers of the grains predominantly existing in insoluble bound form.

Wild rice (*Zizania. Aquatic* L.) as the only cereal indigenous to the Northern American was historically consumed by Native Americans as a staple food (Moyle 1944; Lorenz 1981a; Lorenz 1981b; Steeves 1952). In modern time, it is commercialized and widely used in gourmet food products because of its unique flavour, color, and texture (Oelke et al 1997). The nutritional quality of wild rice tends to be comparable with other cereals characterized by a high content of protein and a low fat content. As a whole grain, wild rice is also a good source of dietary fibre. As reported by Bunzel et al (2002; 2003), several monomeric phenolic acids and dihydrodimers of ferulic and sinapic acids (diferulates and disinapates) were detected and structurally identified in wild rice dietary fibre by using GC-MS and two–dimensional NMR. However, phenolic acids in wild rice whole grain have not been reported. In whole grains, phenolic acids are present in free, soluble esterified and insoluble bound forms (Sosulski, Krygier, & Hogge, 1982). Those reported in wild rice dietary fibre mainly constitute insoluble bound phenolic acids. Free and soluble esterified phenolic acids in wild rice whole grain have not been investigated. Moreover, the antioxidant activities of soluble and insoluble fractions of phenolic acids in wild rice are unknown.

The objectives of the present investigation were (1) to determine the antioxidant activities of crude methanol extracts and alkaline hydrolyzed fractions of both soluble and insoluble phenolic acids; and (2) to identify and quantify the soluble and insoluble phenolic acids in commercial wild rice whole grain.

2.2. Materials and methods

2.2.1. Samples

Eleven commercial wild rice samples were used in this study. The dehulled samples were divided into three categories as follows:

(1) 1 mixed sample: 3 blend mix wild rice consisting of wild rice, white rice, and white basmati was obtained from Kagiwiosa Manomin Inc. (Dinorwic, Ontario).

(2) 1 processed sample: quick-cooking wild rice, already pre-cooked and dehydrated, was obtained from Gourmet House of Riviana Food Inc. (Houston, Texas).

(3) 9 raw samples: three samples, Manomin, large size, and small size wild rice, were purchased from Kagiwiosa Manomin Inc. (Dinorwic, Ontario) and 6 others, A black, B black, C scarified, Canadian lake, hand-harvested, and Minnesota cultivated wild rice, were obtained from Gourmet House of Riviana Food Inc. (Houston, Texas).

White rice purchased from Superstore market (Winnipeg, MB, Canada) was used as a control sample for comparative purposes.

2.2.2. Chemicals

Folin-Ciocalteau reagent, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein (FL), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox) and phenolic acid standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

HPLC grade acetone and methanol were used in the extraction. MS grade water, acetonitrile and acetic acid were used in LC-MS analysis. All the HPLC grade and MS grade solvents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2.3. Sample preparation

Rice grains (white and wild rice) were ground into fine flour by using a Cyclone Sample Mill (Model 3010-018, Udy Corporation, Fort Collins, CO, USA) through a 0.5 mm sieve screen and stored at -20 °C before analysis.

2.2.4. Determination of moisture content of samples

The milled rice flour was weighed before extraction and then dried at 100 °C in an oven. After drying overnight, the dried rice flour was weighed again. All samples were analyzed in triplicate.

2.2.5. Extraction

2 g of rice flour was extracted with 80% methanol twice at a ratio of 20:1 (v/w). Each time, the mixture was kept on a mechanical shaker (Thermo / Lab-Line / Barnstead MAX Q 4000, Artisian Scientific, Champaign, IL, USA) for 1 h at room temperature. After centrifuging (Model 2C5C, MANDEL, Guelph, Ontario) at 4000 rpm for 5 min, the supernatants obtained from each time were combined and concentrated to dryness by using a rotary evaporator (Bochi R-205, Flawil, Switzerland) at 35 °C. The dried methanol extract was redissolved in 5 mL of 50 % methanol and used as crude extracts.

The residue obtained from crude extraction was washed with 40 mL of distilled water to eliminate organic solvent, and then filtered through a Whatman No. 1 filter paper. After drying in a hood at room temperature, the dried residue was kept in a sealed container at 4 °C before subjecting to alkaline hydrolysis. All analyses were performed in duplicate.

2.2.6. Alkaline hydrolysis

The crude methanol extract and the residue remaining from the extraction were separately hydrolyzed with 40 mL of 4 M NaOH on a shaker (Thermo / Lab-Line / Barnstead MAX Q 4000, Artisian Scientific, Champaign, IL, USA) under nitrogen gas for 4 h. After digestion, the solution was adjusted to a pH 1.5~2.0 with 6 M HCl and then extracted with 70 mL of ethyl acetate three times. The combined ether acetate fractions were evaporated to dryness and reconstituted in 2 mL of 50 % methanol. The alkaline hydrolyzed product derived from crude methanol extract was referred to as soluble fraction, and the one obtained from residues was used

as insoluble fraction. Both fractions were directly subjected to total phenolic determination and antioxidant activity measurement. Prior to HPLC analysis, they were filtered through a 0.45 μm syringe filter.

2.2.7. Determination of total phenolic content

The total phenolic content of methanol extract was evaluated by using modifications of the Folin-Ciocalteau method (Singleton & Rossi, 1965). Briefly, 200 μL of the properly diluted crude extracts (or fractions) were firstly mixed with freshly made 1.8 mL of 10-fold diluted Folin-Ciocalteau reagent and then added to 60 g/L of 1.8 mL of sodium carbonate. After reacting for 90 min, the absorbance of the mixture was measured at 725 nm. Gallic acid and ferulic acid were both used for calibration. Results were expressed as mg of gallic acid equivalent (GAE) and ferulic acid equivalent (FAE) per kg of rice on dry weight basis.

2.2.8. Determination of DPPH radical scavenging activity

This assay was based on the method of Brand-William (1995) as modified by Li et al. (2007). Briefly, a 200 μL of appropriately diluted crude extract (or fractions) was added to 3.8 mL of freshly made DPPH radical solution (60 μM). After 60 min of incubation at room temperature, the absorbance at 515 nm was measured. DPPH free radical scavenging activities in crude methanol extracts and different fractions were expressed as μmol of Trolox Equivalents (TE) per 100 g of rice (dry weight basis).

2.2.9. Evaluation of oxygen radical absorbance capacity (ORAC)

The ORAC assay was based on the method described by Huang et al. (2002) and modified by Li et al. (2007). A Precision 2000 automated microplate pipetting system (BIO-TEK Instruments, Inc., Winooski, VT) was used for plate-to-plate transfer of solutions. An FL×800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT) controlled by software KC4 3.0 (version 29) was used with fluorescence filters for an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm.

Firstly, 120 μL of fluorescence working solution was automatically transferred to a 96-well flat bottom polystyrene microplate (Corning Incorporated, Corning, NY, USA) and used as the substrate. Secondly, 20 μL each of buffer solution (blank), Trolox (standard control), appropriately diluted samples and catechin (sample control) were added to the designated wells, respectively. After 20 min incubation at 37 °C, 60 μL of freshly made AAPH solution was added to each well to generate peroxyl radicals. The total reaction time was 50 min. The fluorescence of the reaction mixture was recorded every minute.

The area under the fluorescence decay curve (AUC) was calculated according to the following equation:

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

where f_0 = initial fluorescence reading at 0 min and f_i = fluorescence reading at time i min. Final ORAC values were calculated as follows and expressed as μmol TE/100 g of rice (dry weight basis):

$$ORAC\ \ value = \left[\left(AUC_{sample} - AUC_{blank} \right) / \left(AUCtrolox - AUC_{blank} \right) \right] \times dilution\ \ \ factor$$

2.2.10. HPLC-MS/MS analysis

The chromatographic separation was carried on an HPLC (Waters 2695) equipped with a photodiode array detector (PDA) (Waters 996) and autosampler (Waters 717 plus) (Waters, Milford, MA). The analytical column was a 150 mm × 4.6 mm, 5 μm RP 18 column (Gemini, Phenomenex, USA). The mobile phase consisted of A (0.1% acetic acid in high-purity water) and B (methanol 0.1% acetic acid in methanol). A 75 min-linear gradient was programmed as follows: 0-7 min, 15-20% B; 7-8 min, 20-15% B; 8-20 min, 15% B; 20-21 min, 15-24%B; 21-33 min, 24 % B; 33-34 min, 24-13% B; 34-36 min, 13% B; 36-37 min, 13-20 % B; 37-45 min, 20 % B; 45-46 min, 20-42% B; 46-62 min, 42 % B; 62-63 min, 42-100 % B; 63-68 min, 100 % B; 68-69 min, 100-15% B; 69-75 min, 15 % B. A 10-μL sample solution was injected, and the flow rate was 0.7 mL/min. Full mass spectra were recorded in negative mode by using the capillary voltage of 1.2 kV and cone voltage of 45 V. The flow rate of desolvation gas (N_2) and cone gas (He) were 900 L/h and 50 L/h, respectively. The desolvation temperature and the source temperature were set at 350 °C and 150 °C, respectively. The MS/MS spectra were acquired by using collision energy of 20 V for dimers.

The identification of monomeric phenolic acids in the samples were achieved by comparing retention times, UV maximum absorption and ESI-MS spectra with external standards. To quantify individual phenolic acids, the monitor wavelength was set at 280 nm for hydroxybenzoic acid derivatives and 320 nm for hydroxycinnamic acids and their derivatives.

The quantitative determination of ferulic acid dehydrodimers was accomplished by using *trans*-cinnamic acids as the standard. The response factors (RF) of ferulic acid dehydrodimers against *trans*-cinnamic acids were summarized by Waldron et al (1996) as follows RF (8-8' DiFA) = 0.17, RF (5-5' DiFA) = 0.21, RF (8-O-4' DiFA) = 0.14; RF (8-5' benzofuran form DiFA) = 0.12. Owing to the lack of the response factors for disinapic acids, sinapic acid dehydrodimers were quantified by using sinapic acid equivalent.

2.2.11. Statistical analysis

The results were reported as mean ± standard deviation (SD). Data were analyzed by a one-way analysis of variance (ANOVA) test using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Least significant differences (LSD) at $p < 0.05$ were tested to assess significant differences in antioxidant properties among samples.

2.3. Results and discussion

2.3.1. Total phenolic content (TPC) of crude methanol extracts

According to Kim et al (2006) and Zieliski et al (2000), 80 % aqueous methanol gave the highest yield of extractable phenolic compounds, thus it was considered suitable for extraction of soluble phenolics from wild rice. The total phenolic content (TPC) in crude methanol extracts was measured by Folin-Ciocalteau method. In general, gallic acid is the recommended reference standard. However, in cereal grains, the predominant phenolic acid is ferulic acid, but not gallic

acid. Therefore, ferulic acid was used as another standard. The TPC values are expressed as mg of gallic acid equivalent (GAE) and ferulic acid equivalent (FAE) per kg of rice.

Table 2.1. Moisture content and total phenolic content (TPC) of crude methanol extracts of white rice and wild rice[a]

Class	Sample	Moisture (%)	TPC (mg/ kg)	
			FAE[b,d]	GAE[c,d]
Control	White rice	10.93 ± 0.06	319 ± 9e	46 ± 1e
Mixed	3 blend mix	8.06 ± 0.10	1642 ± 43d	253 ± 7d
Processed	Quick cooking	3.21 ± 0.06	2299 ± 115c	353 ± 18c
Raw	A black	8.00 ± 0.10	2893 ± 145b	445 ± 23b
	B black	6.69 ± 0.06	3802 ± 171a	588 ± 27a
	C scarified	5.36 ± 0.09	3745 ± 151a	579 ± 24a
	Canadian Lake	7.12 ± 0.09	2727 ± 96b	419 ± 15b
	Hand harvested	7.06 ± 0.04	2726 ± 90b	419 ± 14b
	Minnesota cultivated	8.88 ± 0.02	3654 ± 197a	565 ± 31a
	Manomin	6.68 ± 0.23	3784 ±134a	585 ± 21a
	Large size	7.31 ± 0.24	2843 ± 66b	444 ± 7b
	Small size	6.82 ± 0.20	2885 ± 44b	438 ±10b

[a] Mean ± standard deviation (n=6).

[b] Ferulic acid equivalent.

[c] Gallic acid equivalent.

[d] Least significant difference level of probability (p<0.05). Sample means with the same letter in the same column are not significantly different.

As seen in Table 2.1., the TPC measured using the two standards differ in magnitude with FAE being six times higher than GAE. Such significant differences arise from the nature of the molecular structures of ferulic acid and gallic acid. The principle of the Folin-Ciocalteau assay is that when phenolic compounds or other reducing species react with the Folin-Ciocalteau reagent, a blue complex is formed. The absorbance of the blue complex is usually proportional to the number of reacting phenolic hydroxyl groups (Karadag, Ozcelik, & Saner, 2009). Therefore, the molecular structures of standards used for calibration are determinants of the final results recorded. Gallic acid possessing three hydroxyl groups is highly reactive and gives a high absorbance, resulting in low values being measured for the samples. Compared with gallic acid, ferulic acid only has one hydroxyl group, thus causing higher values for the same samples. However, no matter the type of standards chosen for the calibration, significant differences in TPC were detected between white rice (control sample) and wild rice, as well as among different classes of wild rice.

Compared with white rice, wild rice contained more phenolic compounds. The TPC of raw wild rice samples (419 to 588 mg GAE/kg or 2726 to 3802 mg FAE/kg) were 9 to 13 times higher than that of white rice (46 mg GAE/kg or 319 mg FAE/kg). The highest value was found in B black wild rice with the lowest value being observed in Canadian lake wild rice and hand-harvested wild rice. The TPC (253 mg GAE/kg or 1642 mg FAE/kg) recorded for mixed wild rice ranked between white rice and raw wild rice because the endogenous phenolics were diluted by blending white rice and white basmati with wild rice. Significant differences in TPC were also detected between raw and processed wild rice ($p<0.05$). Quick cooking wild rice as the only precooked and dehydrated sample, displayed a significantly lower level of TPC (353 mg GAE /kg or 2299 mg FAE/kg) than uncooked samples (raw wild rice), implying that cooking has a

substantially adverse effect on endogenous phenolic compounds. Besides cooking, other treatments such as soaking and drying also contributed to the reduced phenolic contents in quick cooking wild rice. As reported by Towo et al (2003) and Finocchiaro (2007), water soaking is able to release a portion of soluble phenolic compounds from cereals. The destruction or transformation of phenolic chemical structures during heat treatment may also be responsible for the reduced levels of phenolic compounds (Li, Pickard, & Beta, 2007).

2.3.2. Antioxidant activity (AOA) of crude methanol extracts

Due to the complexity of food systems, no single assay is able to accurately reflect all the individual antioxidants in one reaction system. Thus, utilizing multiple assays to evaluate antioxidant activity tend to be necessary and may provide exclusive information on their multiple abilities to scavenge different radicals. To date, many methods have been developed to measure antioxidant capacity by utilizing different oxidant and target/probe species and diverse reaction conditions. The major two inactivation mechanisms involved in these methods are hydrogen atom transfer (HAT) mechanism and single electron transfer (SET) mechanism (Prior, Wu, & Schaich, 2005). Oxygen radical absorbance capacity (ORAC) assay and DPPH radical scavenging activity assay as the representatives of these two mechanism based methods were conducted in our study.

ORAC is a HAT based assay measuring the antioxidant capacity against peroxyl radicals (Huang, Ou, & Prior, 2005; Karadag, Ozcelik, & Saner, 2009). Due to the reaction condition (37 °C, pH=7) most relevant to human biology, this assay has been suggested as a standard

method for assessing food antioxidant capacity (Prior, Wu, & Schaich, 2005). The ORAC values estimated for white and wild rice are shown in Figure 2.2 and expressed as μmol of trolox equivalent (TE) per 100 g of rice.

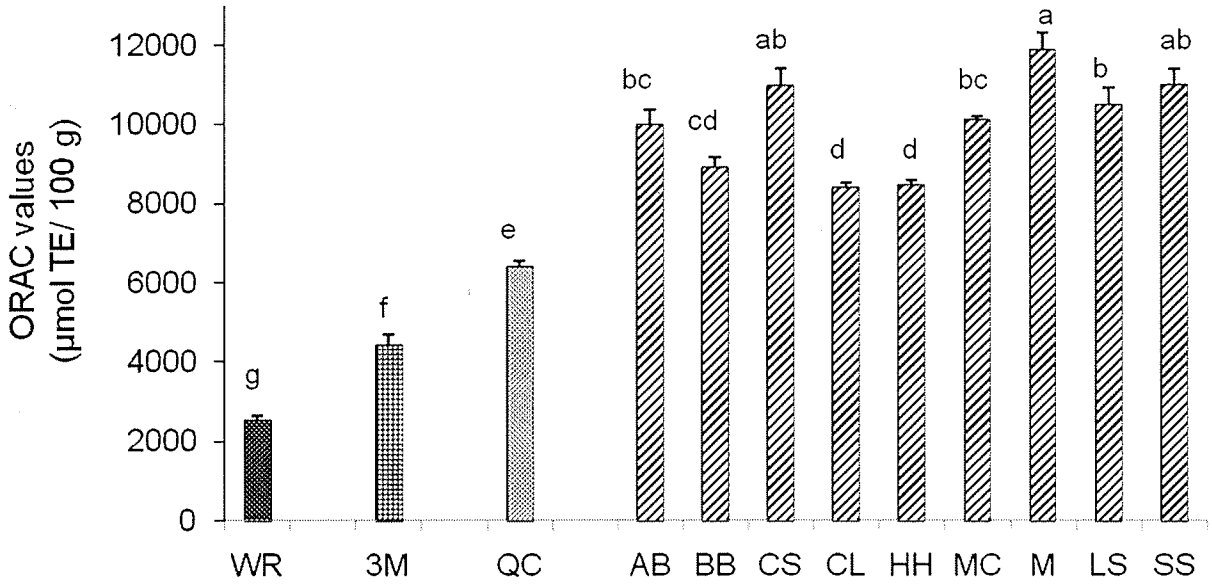


Figure 2.2. ORAC values of crude methanol extracts from white rice and wild rice. WR, white rice; 3M, 3 blend mix; QC, quick cooking; AB, A black; BB, B black; CS, C scarified ; CL, Canadian lake; HH, hand harvested; MC, Minnesota cultivated ; M, Manomin; LS, large size; SS, small size. The vertical bars represents the standard deviation (n=6). Columns marked by different letters are significantly different ($p < 0.05$).

Among all the samples, the white rice control had the lowest ORAC value (2534 μmol TE/100 g). The second lowest value was found in mixed wild rice (4454 μmol TE/100 g). For raw wild rice samples, the ORAC values varied from 8346 to 11903 μmol TE/100 g, with the highest and lowest values observed in A black wild rice and hand harvested wild rice, respectively. Significant differences in ORAC values among raw wild rice samples are most likely attributable to the cultivars, growing and harvesting conditions, and post-harvest processing. The precooked sample, quick cooking wild rice had a lower ORAC value (6430 μmol TE/100 g) than uncooked samples indicating that cooking substantially impairs wild rice antioxidant activity.

Compared with ORAC assay, DPPH method measures antioxidant inhibition of nitrogen radical induced oxidation (Karadag, Ozcelik, & Saner, 2009). Although originally considered as a single electron transfer (SET) reaction (Brand-Williams, Cuvelier, & Berset, 1995; Foti, Daquino, & Geraci, 2004), practically it may involve with both mechanisms (SET & HAT) (Karadag, Ozcelik, & Saner 2009; Sanchez-Mareno, 2002). As seen in Figure 2.3, wild rice exhibited significantly higher DPPH radical scavenging activities than white rice. The DPPH radical scavenging activities of raw wild rice samples were 2~3 times greater (388 to 541 μmol TE/100 g) than that of white rice (159 μmol TE/100 g). Mixed wild rice was expected to rank between white and raw wild rice (318 μmol TE/100 g). Consistent with ORAC results, decreased DPPH radical scavenging capacity (347 μmol TE/100 g) were also found in processed samples.

Comparing the results obtained from these two assays, although the final values were different, similar conclusions could be reached. Wild rice had higher antioxidant activity than white rice, indicating a better phytochemical profile of the former than the latter.

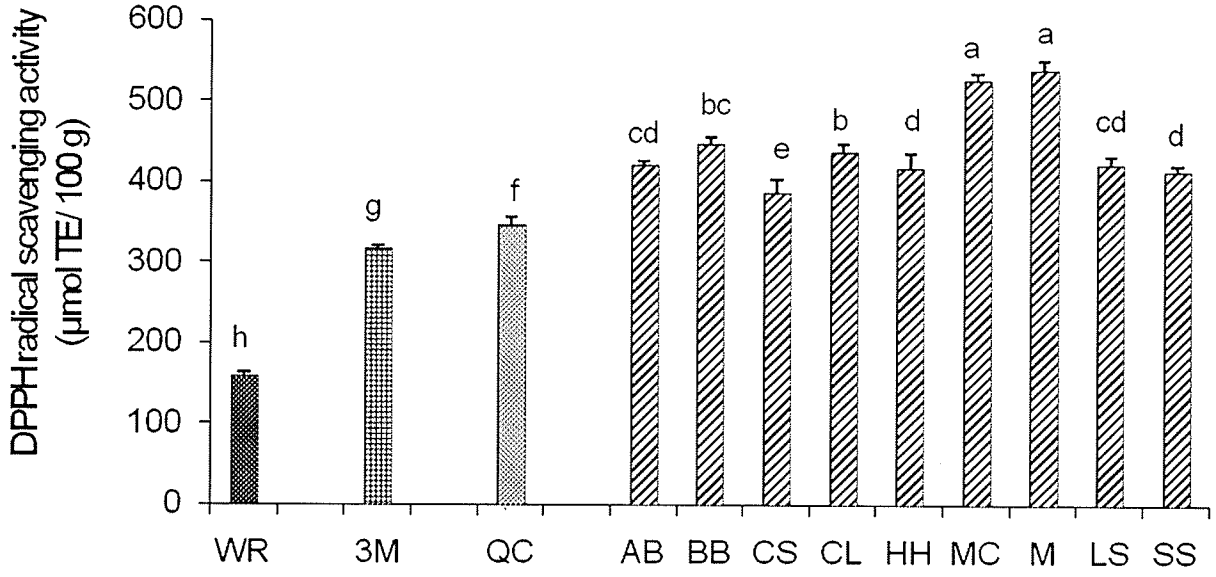


Figure 2.3. Free radical scavenging activity of crude methanol extracts from white rice and wild rice on DPPH radical (at 60 min). WR, white rice; 3M, 3 blend mix; QC, quick cooking; AB, A black; BB, B black; CS, C scarified ; CL, Canadian lake; HH, hand harvested; MC, Minnesota cultivated ; M, Manomin; LS, large size; SS, small size. The vertical bars represents the standard deviation (n=6). Columns marked by different letters are significantly different ($p < 0.05$).

2.3.3. Total phenolic content and antioxidant activity of soluble and insoluble fractions

Soluble and insoluble fractions are alkaline hydrolyzed products, respectively obtained from crude methanol extracts and the residues remaining after methanol extraction. The total phenolic content (TPC) in both fractions are shown in Table 2.2, expressed as mg of gallic acid equivalent per kg of rice on dry basis.

Table 2.2. Total phenolic content (TPC) in soluble and insoluble fractions of white rice and wild rice[a]

		TPC (GAE[b] mg/ kg)		
Class	**Sample**	**Soluble**	**Insoluble**	**∑TPC**
Control	White rice	12.34±0.45	31.57±1.62	43.91
Mixed	3 blend mix	53.08±2.82	140.25±2.84	193.33
Processed	Quick cooking	88.60±1.90	142.75±2.33	231.35
Raw	A black	137.06±3.65	164.01±3.49	301.07
	B black	164.68±4.85	189.79±2.53	354.47
	C scarified	161.48±2.84	206.61±9.95	368.09
	Canadian Lake	105.66±3.06	166.01±2.42	271.67
	Hand harvested	106.04±1.53	158.55±2.18	264.58
	Minnesota	153.81±2.92	186.44±3.60	340.25
	Manomin	169.19±3.52	212.08±8.08	381.28
	Large size	124.79±4.04	168.87±2.14	293.66
	Small size	124.02±4.44	172.39±2.82	296.41

[a] Mean ± standard deviation (n=6).

[b] Gallic acid equivalent.

In general, the insoluble fractions contained more phenolic compounds than the soluble fractions for the same sample. For white rice, the phenolic content in insoluble fraction is twice as high as the soluble fraction. However, the differences in phenolic contents of different fractions from wild rice were not as high as in white rice.

For soluble fractions, the phenolic contents in raw wild rice samples ranged from 106 mg to 169 mg GAE/kg. The highest and lowest values were respectively found in Manomin wild rice and hand harvested wild rice. The processed quick cooking wild rice had TPC of 89 mg GAE/kg. A relatively low TPC was also detected in mixed wild rice (53 mg GAE/kg). In comparison with wild rice, white rice control contained the lowest phenolic compounds with a level of 12 mg GAE/kg.

With regard to insoluble fractions, raw wild rice samples still exhibited the highest phenolic contents followed by processed and mixed wild rice samples. Among raw samples, the phenolic contents varied from 158 to 212 mg GAE/kg. The TPC in processed and mixed wild rice samples was 143 and 140 mg GAE/kg, respectively. White rice displayed the lowest TPC (32 mg GAE/kg).

The sum of total phenolic contents present in soluble and insoluble fraction ($\sum$TPC) for each sample was lower than the corresponding value of the crude methanol extract. The crude methanol extracts not only comprised simple phenolic acids, but also contained a wide range of polyphenols. After treatment with NaOH, and a sequence of acidification and re-extraction with ethyl acetate, flavonoids and other neutral polyphenols were partially removed (Robbins 2003). Thus phenolic acids were the predominant components in the soluble and insoluble fractions. Even though alkaline hydrolysis was able to release some ester- or ether-linked phenolic acids

from the cell wall, the amount of released phenolic acids was less than that of polyphenols present in crude methanol extracts. Therefore, the hydrolyzed products (soluble and insoluble fractions) contained less TPC than methanol extracts. The sum of TPC in the soluble and insoluble fractions ($\sum$TPC) in wild rice samples was approximately 5 times higher than that of white rice. Significant differences in $\sum$TPC were also detected among mixed, processed and raw wild rice samples.

Consistent with the findings on TPC, the antioxidant activity of insoluble fractions was also higher than that of soluble fractions (Figure 2.4.). Compared with DPPH free radical scavenging activity of crude methanol extracts, the hydrolyzed fractions displayed much lower antioxidant activity, an indication that phenolic acids are only a small portion of antioxidant compounds in wild rice. Phenolic compounds such as flavonoids and other phytochemicals may contribute to the bulk antioxidant capacity of wild rice (Qiu, Liu, & Beta, 2009).

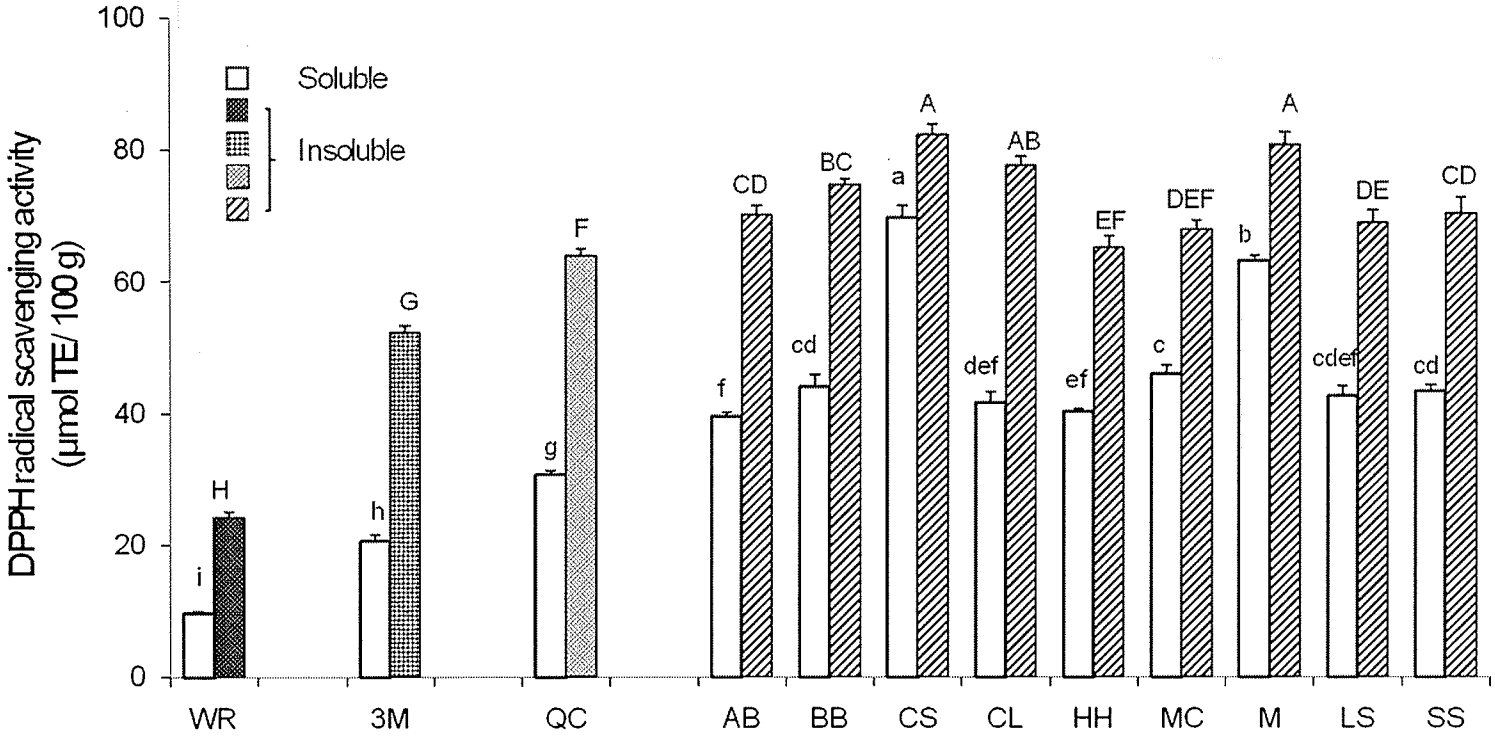


Figure 2.4. DPPH radical scavenging activity in soluble and insoluble fractions (at 60 min). WR, white rice; 3M, 3 blend mix; QC, quick cooking; AB, A black; BB, B black; CS, C scarified ; CL, Canadian lake; HH, hand harvested; MC, Minnesota cultivated ; M, Manomin; LS, large size; SS, small size. The vertical bars represents the standard deviation (n=6). Columns marked by different letters are significantly different ($p < 0.05$).

2.3.4. Identification and quantification of monomeric phenolic acids

Figure 2.5 shows the UV spectra of selected phenolic acid standards. It can be seen that different phenolic acids displayed various UV profiles. Distinguished UV spectra were detected between hydroxybenzoic acids and hydroxycinnamate group. The typical UV absorption of hydroxybenzoic acids was in the range of 210 nm to 310 nm, whereas hydroxycinnamic acids have a characteristic UV absorption around 320 nm. Due to this attribute, the detection of phenolic acids is usually accomplished by setting monitor wavelengths at 280 nm and 320 nm (Robbins, 2003).

Figure 2.6 (A) and (B) are the LC chromatograms of phenolic acid standards, which were recorded at 280 nm and 320 nm, respectively. It was evident that phenolic acids with benzoic acid carbon framework were absent in Figure 2.6 (B), indicating that the wavelength of 320 nm was not able to detect all the phenolic acids. Compared with Figure 2.6 (B), all the standards were present in Figure 2.6 (A). Therefore, in order to reflect all the phenolic acid constituents, the LC chromatograms of wild rice samples (Figure 2.7) was recorded at 280 nm.

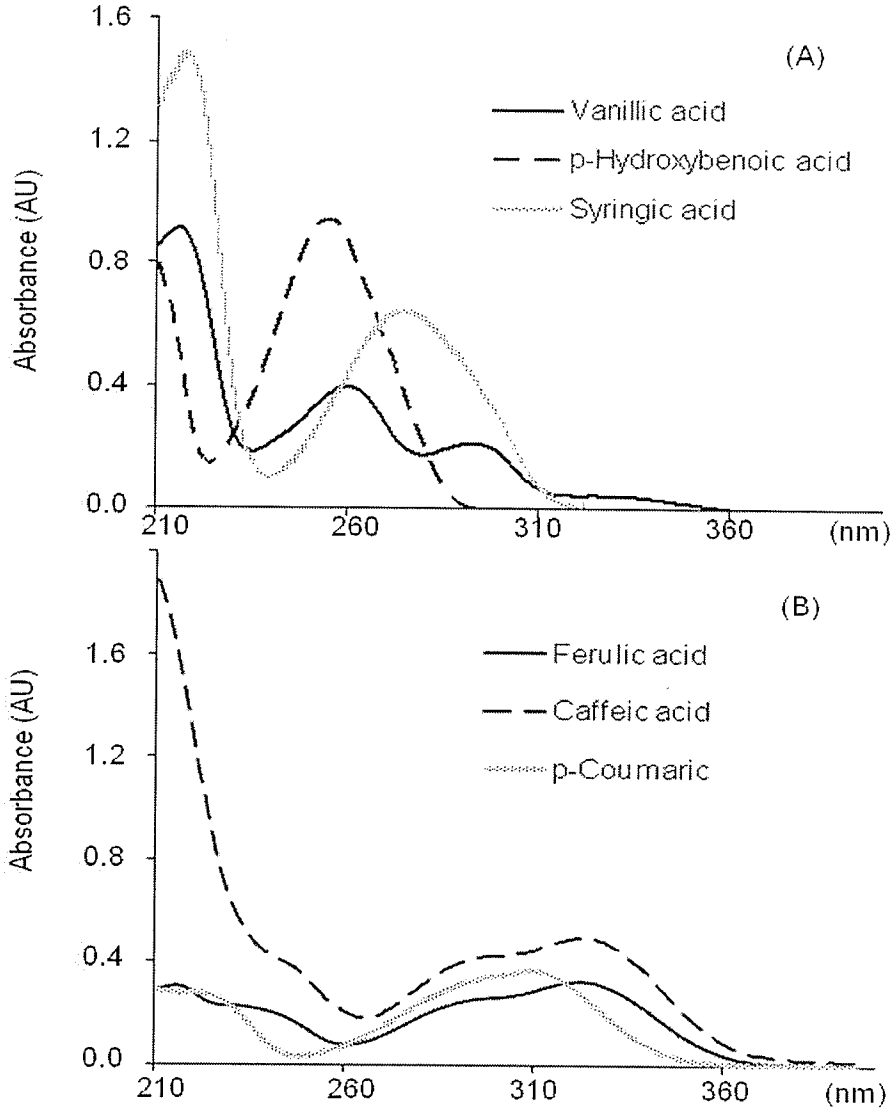


Figure 2.5. UV spectra (210-400 nm) of selected phenolic acid standards within two subgroups (A) hydroxybenozic acids: vanillic, *p*-hydroxybenoic, and syringic acids; (B) hydroxycinnamic acids: caffeic, ferulic, and *p*-coumaric acids.

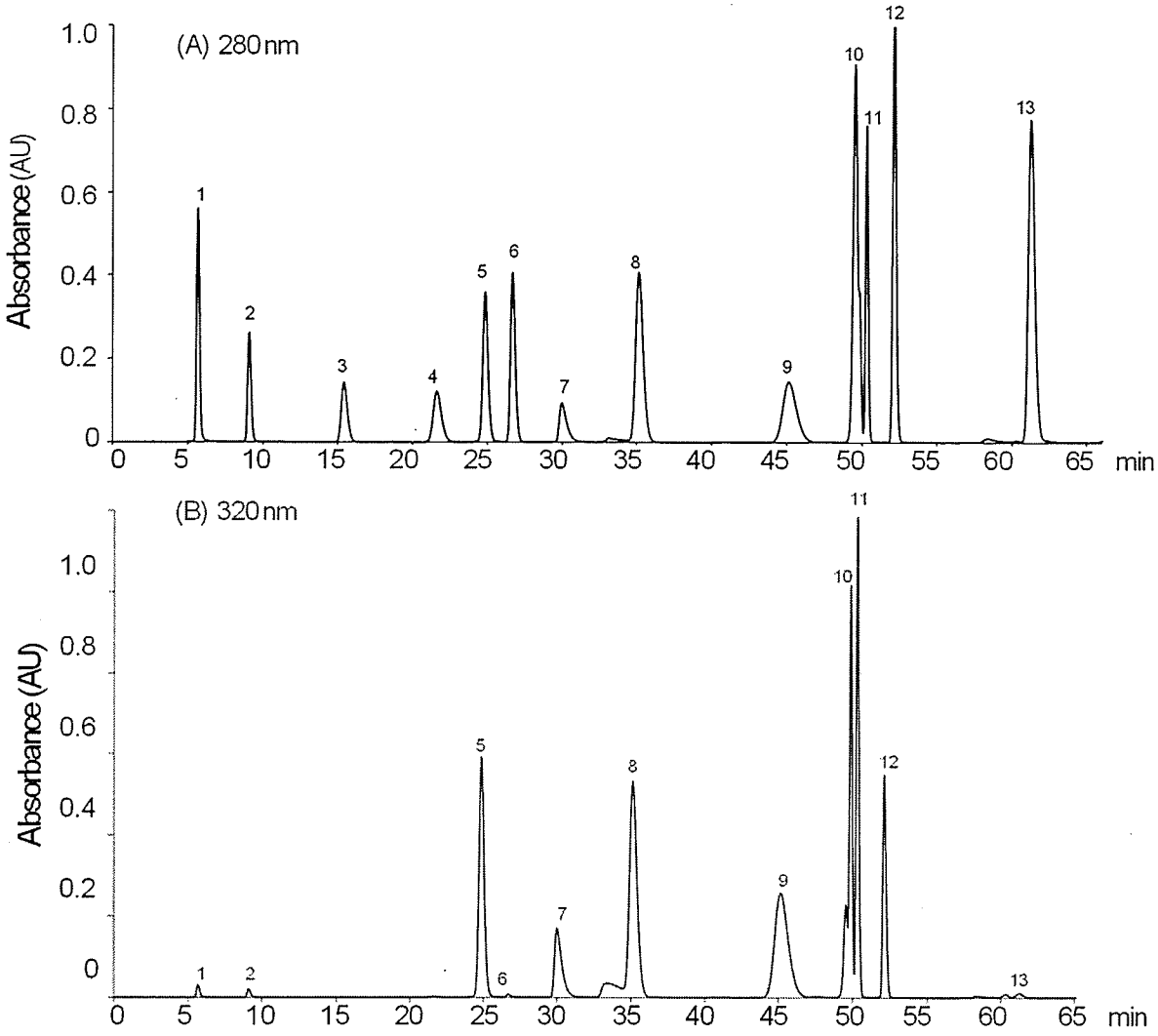


Figure 2.6. LC chromatogram of a standard mixture of phenolic acids with detection (A) at 280 nm for benzoic acid derivatives: 1 gallic acid, 2 protocatechuic acid, 3 *p*-hydroxybenzoic acid, 4 vanillic acid, 5 caffeic acid, 6 syringic acid, 7 chlorogenic acid and 13 ellagic acid; (B) at 320 nm for cinnamic acid derivatives: 8 *p*-coumaric acid, 9 ferulic acid, 10 sinapic acid, 11 *m*-coumaric acid, and 12 *o*-coumaric acid.

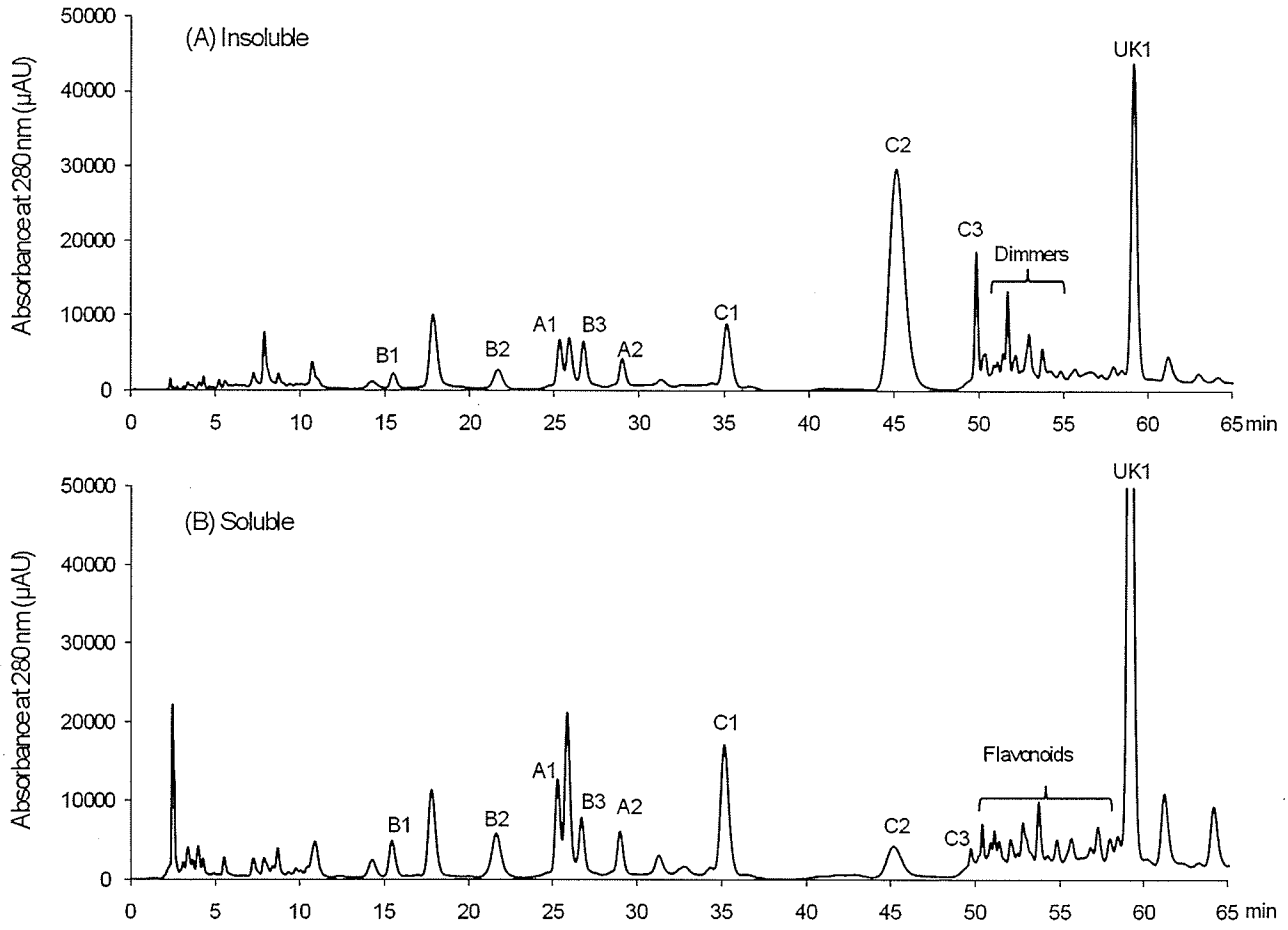


Figure 2.7. LC chromatogram (280 nm) of alkaline hydrolysates from Manomin wild rice: (A) insoluble fraction; (B) soluble fraction.

Figure 2.7 is the LC chromatograms of soluble and insoluble fractions from Manomin wild rice, one of the raw samples. By comparing of retention times and UV absorption with external standards, the primary assignments of hydroxybenzoic acids and hydroxycinnamic acids were accomplished. To distinguish which group the identified peaks belonged to, the letter B was used to represent benzoic acid derivatives and letter C was employed for hydroxycinnamates. Within each group, the peaks were further numbered according to the retention time. Peak B1 was the first phenolic acid eluted from the column with a retention time of 15.42 min, thus assigned as *p*-hydroxybenzoic acid. Peak B2 present at 21.65 min was considered as vanillic acid and peak B3 (26.70 min) corresponded to syringic acid. In addition to these three hydroxybenzoic acids, peaks labelled C1, C2, and C3 referred to as hydroxycinnamic acids were respectively assigned as *p*-coumaric acid, ferulic acid, and sinapic acid. To further confirm the identification, the MS/MS spectra of these peaks (Figure 2.8 and Figure 2.9) were examined. According to the identical parent ion $[M-H]^-$ with reference standards and the typical mass loss (44 Da) observed for phenolic acids, it became evident that the previous assignments were correct. The mass loss of 44 Da was caused by the loss and rearrangement of –COOH group from the parent ion.

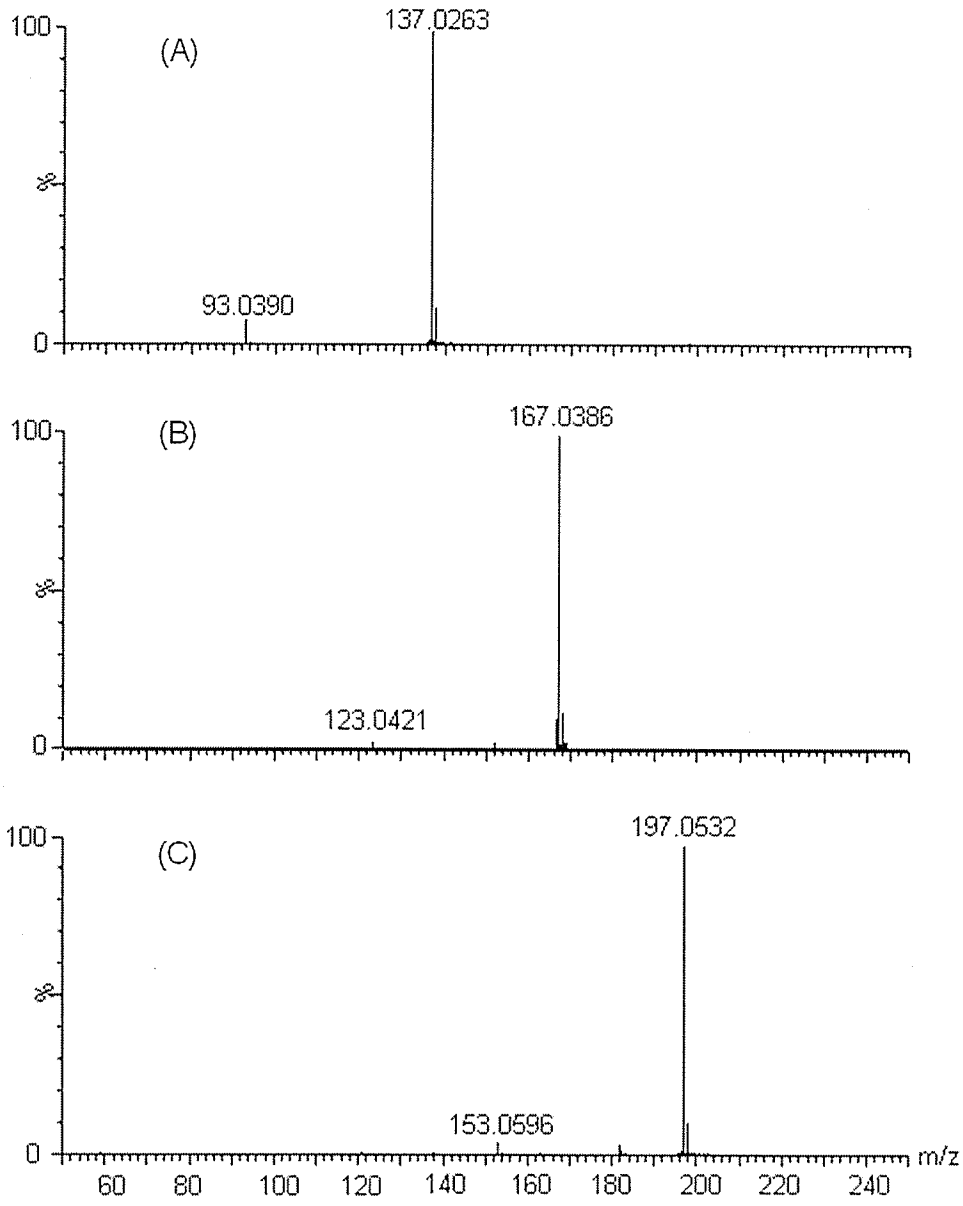


Figure 2.8. Parent ion and typical product ion obtained from (A) peak B1, (B) peak B2, and (C) peak B3 of Manomin wild rice.

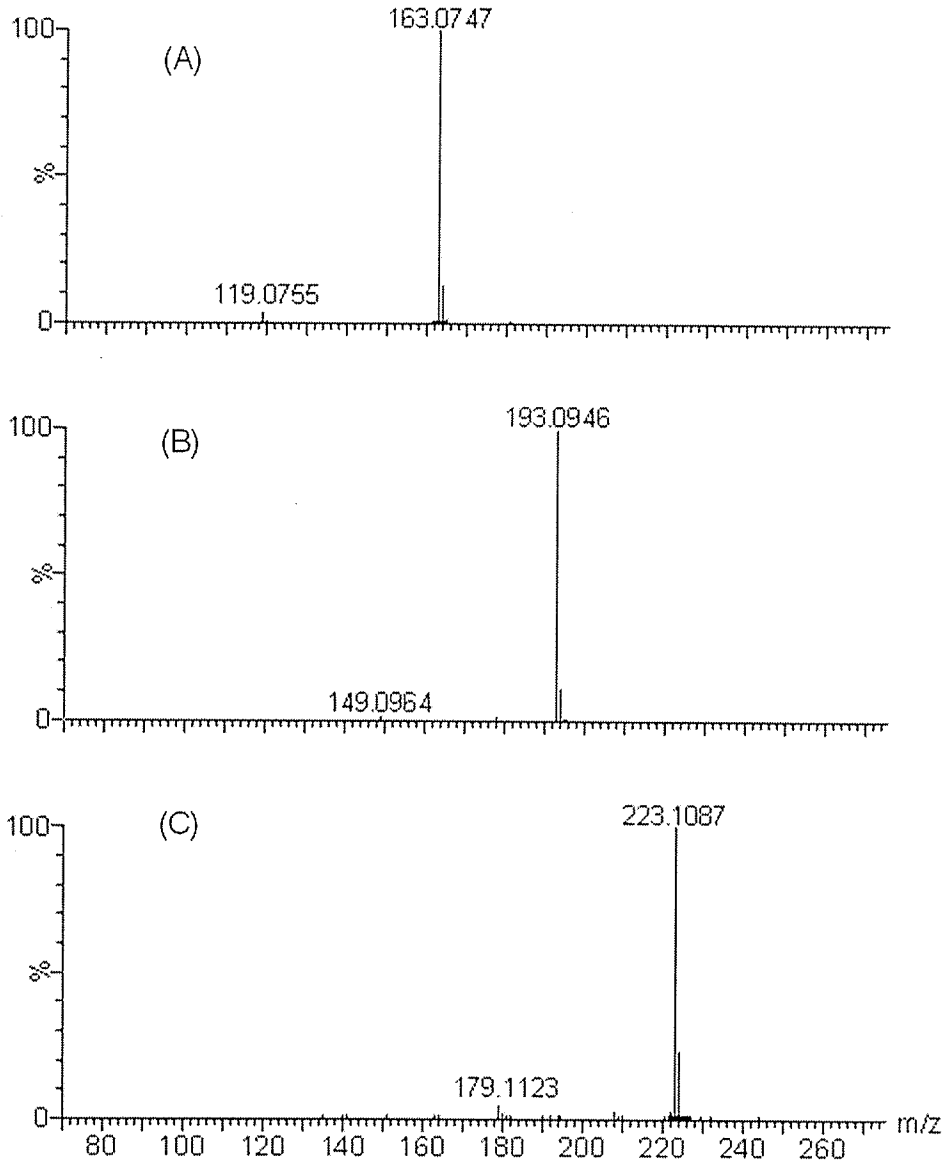


Figure 2.9. Parent ion and typical product ion obtained from (A) peak C1, (B) peak C2, and (C) peak C3 of Manomin wild rice.

In addition to hydroxybenzoic and cinnamic acids, two phenolic aldehydes labelled as A1 and A2 (Figure 2.7) were also identified in wild rice. They were assigned as *p*-hydroxybenzaldehyde and vanillin, respectively. The characteristics of all the identified peaks are summarized in Table 2.3.

Table 2.3. Identification of monomeric phenolic acids in wild rice

Peak	Assignment	t_R (min)	UV (nm)	$[M-H]^-$ *m/z*
	Hydroxybenzoic acids[a]			
B1	*p*-hydroxybenzoic	15.42	255	137
B2	vanillic	21.65	260, 293	167
B3	syringic	26.70	217,277	197
	Hydroxycinnamic acids[a]			
C1	*p*-coumaric	35.13	210, 308	163
C2	ferulic	45.15	215, 321	193
C3	sinapic	50.30	320	223
	Phenolic aldehydes[b]			
A1	4-OH-benzaldehyde	25.02	280	136
A2	vanillin	28.98	211, 304	166

[a] Identification based on comparison with commercial standards

[b] Identification based on comparison with literature data (Waldron et al 1996)

Although protocatechuic acid and its aldehyde analogue were previously reported in wild rice dietary fibre (Bunzel et al 2002), they were not detected neither in insoluble nor in soluble fractions. The possible reason is that the sample preparation and extraction method involved in our study varied from the one stated in the report by Bunzel et al (2002). It is possible that the combination of enzyme, alkaline and acid hydrolysis released more phenolic acids.

The quantitative measurement of soluble and insoluble phenolic acids in wild rice was performed on the basis of the LC chromatogram data relative to the external standards. The quantification wavelengths were set at 280 nm for hydroxybenzoic acid derivatives and 320 nm for hydroxycinnamic acids and their derivatives. Concentration of individual phenolic acids in different fractions of wild rice is shown in Table 2.4 and Table 2.5.

As shown in Table 2.4, ferulic acid, as expected, was the most abundant phenolic acid in the insoluble fractions for all the rice samples (white and wild rice). The highest concentration of ferulic acid found in wild rice was 355 mg/kg. Sinapic acid, although not dominant in white rice, was found in the second highest amounts in wild rice, ranging from 55 to 96 mg/kg for raw samples. Besides ferulic acid and sinapic acid, *p*-coumaric acid was also found in significant quantities (11.51 to 43.50 mg/kg) in wild rice insoluble fractions. Considering that the above three phenolic acids belong to hydroxycinnamic acids, it can be concluded that in the insoluble fractions, hydroxycinnamic acids were the predominant phenolic acids. In contrast to insoluble fractions, the soluble fractions did not contain such high levels of hydroxycinnamic acids (Table 2.5). The contents of ferulic acids in the soluble fractions only ranged from 27 to 59 mg/kg in raw wild rice. Sinapic acid, as the second most abundant phenolic acid in the insoluble fractions, only occurred in trace amounts in the soluble fractions, thus the contents were not shown in

Table 2.5. In spite of the low contents of hydroxycinnamic acids, the soluble fractions contained comparable amounts of hydroxybenzoic acids (*p*-OH-benzoic, vanillic, and syringic acids) as the insoluble fractions. In the insoluble fractions, the hydroxycinnamic acids were the most abundant among the phenolic acids. As expected, ferulic acid was the major hydroxycinnamic acids with concentrations ranging from 241 to 355 mg/kg in raw wild rice samples. Significant amounts of sinapic acid and *p*-coumaric acid were also detected in the insoluble fractions. However, in the soluble fractions, hydroxybenzoic acids were the predominant phenolic acids with vanillic acid in the most quantities. The highest content of vanillic acid in raw samples was 46 mg/kg.

By comparing the total monomeric phenolic acid content in different fractions, it is clearly evident that the majority of phenolic acids in wild rice whole grain were found in the insoluble-bound form, predominantly as cinnamic acid derivatives.

Table 2.4. Contents of phenolic acids in insoluble fractions from wild rice (mg/kg on dry weight basis)[a]

Class	Sample name	*p*-OH-benzoic	Vanillic	Syringic	*p*-Coumaric	Ferulic	Sinapic	8-8' DiSA [b]	8-O-4' DiFA [c]
Control	White rice	nd [d]	1.06±0.28	trace	3.60±0.07	102.01±16.50	trace	nd	trace
Mixed	3 blend mix	2.16±0.24	10.57±1.49	4.97±0.35	11.51±1.04	134.71±15.55	13.06±1.45	4.54±1.32	12.33±2.45
Processed	Quick cooking	8.91±1.02	20.67±2.88	11.16±1.21	27.19±0.02	232.47±14.33	79.37±2.97	12.58±2.47	25.24±1.97
Raw	A black	8.42±1.24	18.10±2.10	9.59±0.58	36.46±0.83	295.20±24.90	73.37±3.31	15.47±2.35	29.45±1.31
	B black	11.90±1.22	30.33±4.03	17.10±2.44	43.50±0.34	345.29±34.34	55.13±2.65	18.78±3.78	30.17±2.65
	C scarified	7.44±0.87	24.24±3.78	13.36±1.68	30.65±0.44	301.08±19.01	56.88±2.04	16.11±2.87	28.78±2.04
	Canadian lake	4.62±0.21	17.25±2.62	9.07±0.58	25.18±0.63	290.66±23.18	96.94±3.47	12.08±1.68	33.00±3.47
	Hand harvested	8.40±0.98	20.56±4.22	11.10±1.23	34.80±2.78	241.58±21.47	82.41±3.47	10.60±1.27	30.46±4.47
	Minnesota	11.61±1.14	21.81±3.48	11.87±0.97	26.56±0.08	278.24±18.52	67.77±3.65	12.03±1.61	30.96±3.65
	Manomin	12.52±0.95	26.25±1.10	14.59±1.94	35.03±0.55	355.41±12.25	81.67±2.38	10.15±2.01	32.52±2.38
	Large size	6.26±0.57	19.53±1.07	10.46±0.55	25.85±1.24	303.13±21.77	91.02±4.75	13.40±1.44	32.39±2.75
	Small size	4.43±0.64	16.17±2.92	8.40±0.69	35.23±0.70	275.71±17.54	68.43±2.94	11.82±1.52	33.58±4.94

[a] Mean ± standard deviation (n=2).

[b] Quantified by using *tans*-cinnamic aid as the standard; the response factor of 8-O-4' DiFA in relation to *tans*-cinnamic aid was 0.14 (Walkdron et al, 1996).

[c] Quantified by using sinapic acid as the standard and expressed as sinapic acid equivalent.

[d] Not detected.

Table 2.5. Contents of phenolic acids in soluble fractions from wild rice (mg/kg on dry weight basis)[a]

Class	Sample name	*p*-OH-benzoic	Vanillic	Syringic	*p*-Coumaric	Ferulic
Control	White rice	nd[b]	1.29±0.28	Nd	3.21±0.15	9.24±1.21
Mixed	3 blend mix	6.01±1.10	8.58±1.49	5.77±0.96	4.85±0.05	16.84±1.02
Processed	Quick cooking	12.01±1.57	33.52±2.88	12.05±1.38	18.57±2.23	20.71±2.79
Raw	A black	21.93±2.68	40.49±6.10	16.90±2.07	29.23±1.72	40.33±3.57
	B black	25.00±2.44	42.12±5.03	15.29±1.58	41.10±3.74	29.05±2.78
	C scarified	46.98±3.58	33.42±3.78	32.53±3.52	52.30±4.42	59.68±2.30
	Canadian lake	16.76±1.01	18.55±2.62	8.76±0.88	33.01±1.92	27.25±1.51
	Hand harvested	25.74±2.34	26.03±4.22	12.58±1.34	26.01±1.38	34.75±2.80
	Minnesota	16.56±1.68	33.60±3.48	12.69±1.33	22.78±2.26	34.66±3.44
	Manomin	22.61±2.30	23.14±1.10	16.50±2.00	36.18±1.51	51.03±4.14
	Large size	11.69±0.69	21.59±1.07	9.99±0.93	22.13±1.13	43.85±3.68
	Small size	19.87±0.35	22.64±2.92	8.96±0.67	25.93±2.19	37.96±2.78

[a] Mean ± standard deviation (n=2).

[b] Not detected.

2.3.5. Identification and quantification of dimeric phenolic acids

Phenolic dehydrodimers as structural components in cell walls play an important role in fortifying cell wall mechanical properties by cross-linking the arabinoxylan chains (Renger & Steinhart, 2000). The first identified phenolic dehydrodimers is dehydrodiferulic acid known to be 5-5' coupled in wheat pentosan (Geissmann & Neukom, 1973). Since 1994, a couple of novel ferulic acid dehydrodimers have been isolated from various cell walls including 8-8' (noncyclic and cyclic), 8-O-4', 8-5' (noncyclic and cyclic), and 4-O-5' coupled dimers. Cereal dietary fibre mainly consisting of cell walls give rise to 8-O-4', 5-5', and 8-5' coupled diferulic acids (Bunzel et al, 2001). Apart from ferulic acid, dimerisation of sinapic acid can also be observed in some cereal dietary fibre (Bunzel et al, 2003). In our study, the alkaline hydrolysate from wild rice residue is composed of cereal dietary fibre. Thus, a variety of phenolic dehydrodimers were expected to be present in the insoluble fractions, but not the soluble fractions.

Figure 2.10 (A) is the LC chromatogram of the insoluble fraction from Manomin wild rice. By plotting typical molecular ions at *m/z*= 385 and 445 expected from DFA and DSA (Figure 2.10 (B) and (C)), four ferulate dehydrodimers (peak DF1 to DF4) and two sinapate dehydrodimers (peak DS1and DS2) were detected in wild rice.

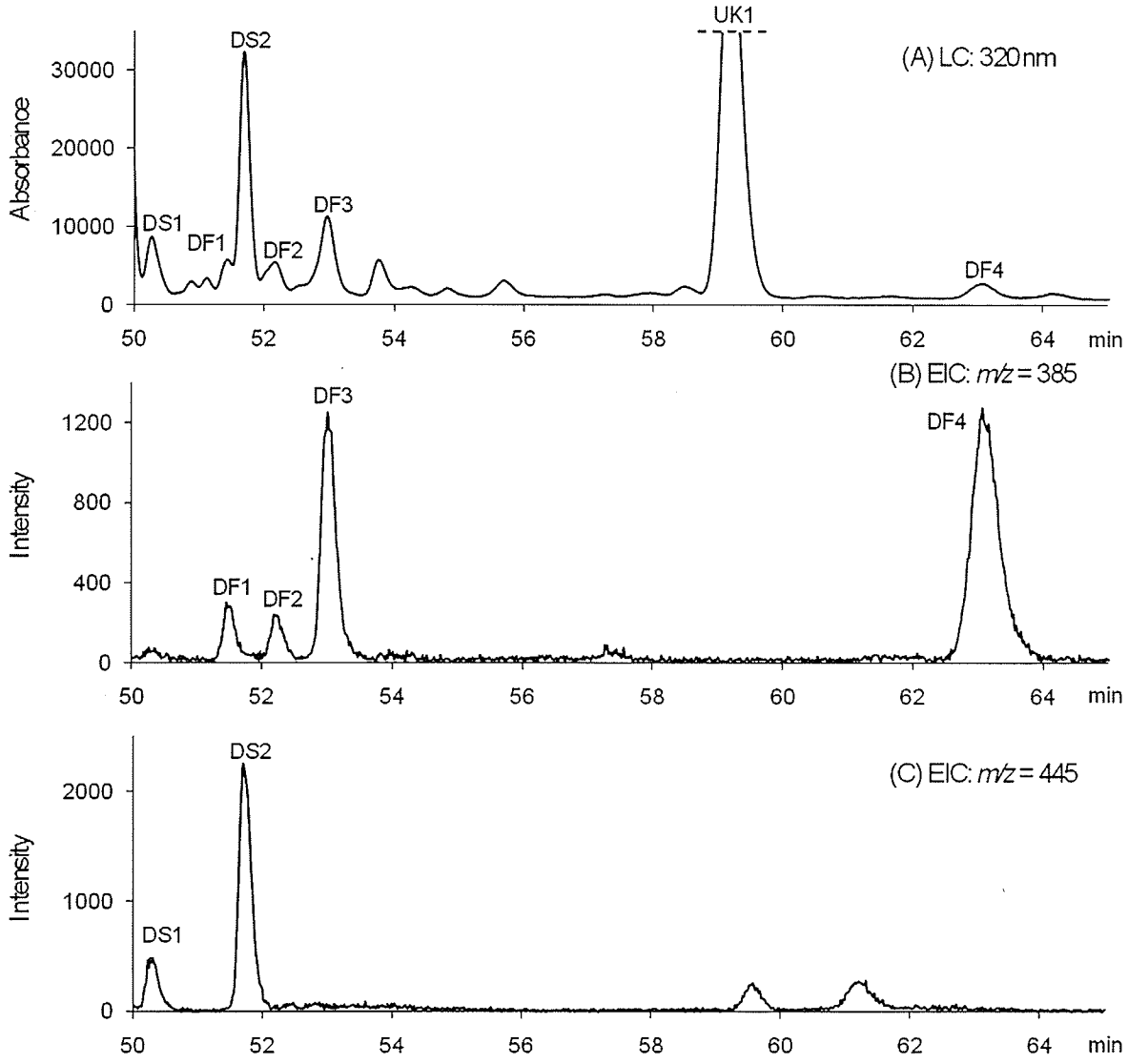


Figure 2.10. (A) LC chromatogram (50-65 min) of insoluble fraction obtained from Manomin wild rice; (B) extracted ion chromatogram (EIC) of ions at *m/z* = 385; (C) extracted ion chromatogram (EIC) of ions at *m/z* = 445.

The identification of chemical structures of individual DFA and DSA were carried out by comparing their retention times, MS spectra and tandem mass (MS/MS) spectra with literature data. The suggested elution sequence of diferulic acids was 8-8' (cyclic or aryltetralin form) DFA < 8-8' (noncyclic or linear form) DFA< 5-5' DFA< 8-O-4' DFA< 8-5' (noncyclic or linear form) DFA <8-5' (cyclic or benzofuran form) DFA (Waldron et al, 1996). Based on the MS/MS spectra of the detected peaks (DF1 to DF4) (Figure 2.11), they were assigned as 8-8', 5-5', 8-O-4', and 8-5' (benzofuran form) coupled DFA, respectively. Peak DF3 appeared as the dominant peak contrary to the other three peaks indicating 8-O-4' DFA as the predominant DFA in wild rice. Table 2.5 summarizes the characteristics of identified dehydrodimers in wild rice, which are arranged according to their relative retention times.

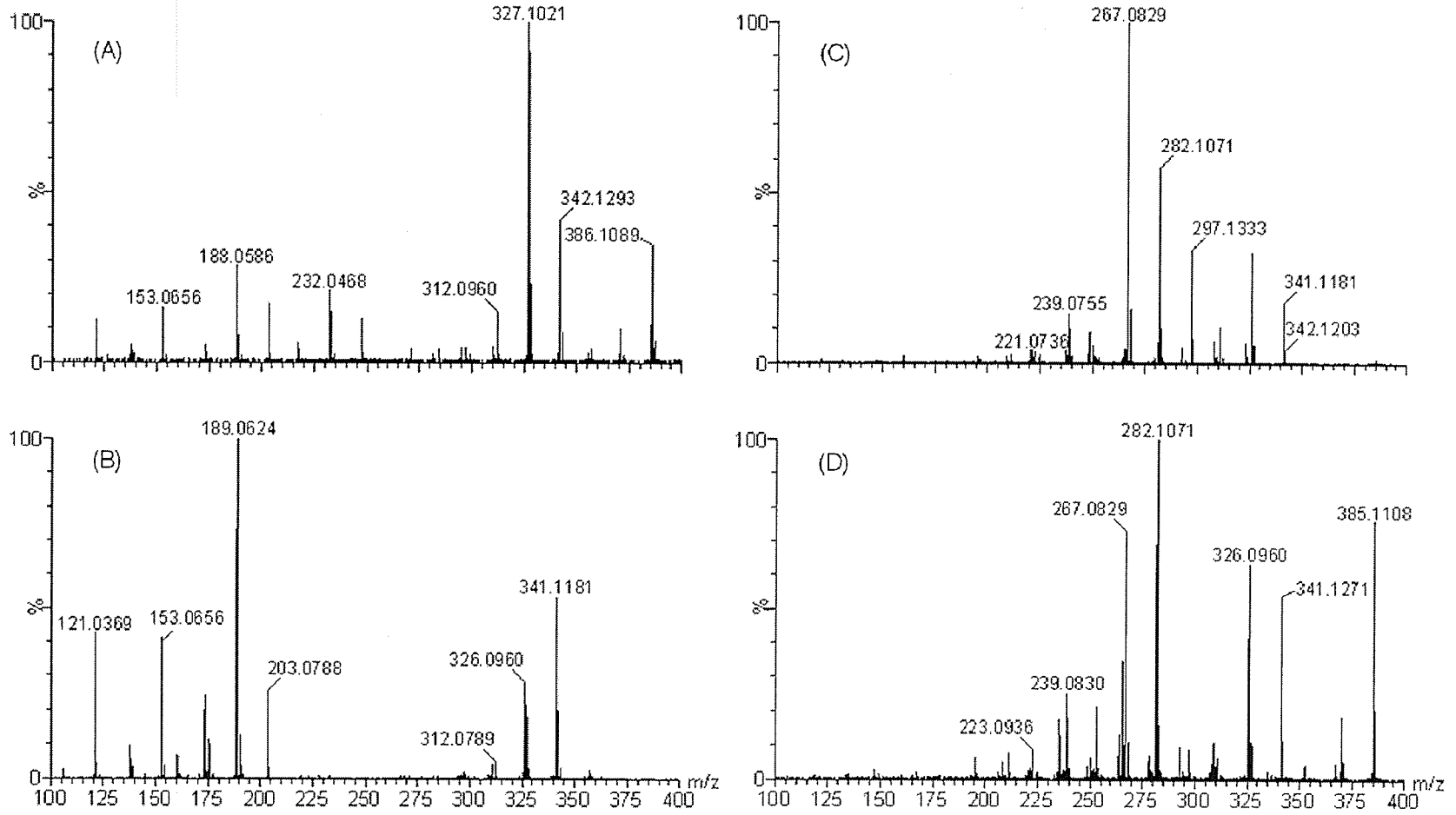


Figure 2.11. MS/MS spectra of diferulic acids (DFA) in wild rice (A) peak DF1 (8-8'); (B) peak DF2 (5-5'); (C) peak DF3 (8-O-4'); and (D) peak DF4 (8-5' benzofuran form).

Table 2.5. HPLC-MS/MS characterization of diferulic acids (DFA) and disinapic acids (DSA) in wild rice insoluble fraction

Peak	Assignment[a]	t_R (min)	$[M-H]^-$ *m/z*	Product ion *m/z*
DS1	8-8' DSA (c)	50.28	445	401*, 342, 327, 312, 232, 188
DF1	8-8' DFA (nc)	51.73	385	342, 327*, 312, 232, 188, 153
DS2	8-8' DSA (nc)	51.68	445	341, 326, 203, 189*, 153, 121
DF2	5-5' DFA	52.31	385	341, 326, 312, 203, 189*, 153, 121
DF3	8-O-4' DFA	53.21	385	341, 326, 311, 297, 282, 267*, 239
DF4	8-5' DFA(c)	63.55	385	370, 341, 326, 282*, 267, 239, 223

* The product ion has the highest ion intensity.

[a] The letter c represents cyclic isomers, whilst nc stands for non-cyclic isomer

Sinapate dehydrodimers can be distinguished from other dehydrodiferulic acids as they only occur as 8-8'-coupled and 8-O-4'-coupled products, with the former as the predominant form (Bunzel et al, 2003). As observed with dehydrodiferulic acids, the cyclic 8-8 isomers are eluted from the column prior to the noncyclic products (Bunzel et al, 2003). Thus, in our study, peak DS1 present at 50.28 min was recognized as 8-8' DSA (cyclic or aryltetralin form) and the peak present at 51.68 min as the noncyclic form of DSA. The MS/MS spectra gave a very clear identification of the chemical structures of these two DSAs (Figure 2.12). 8-O-4' DSA was not detected in the insoluble fraction of wild rice.

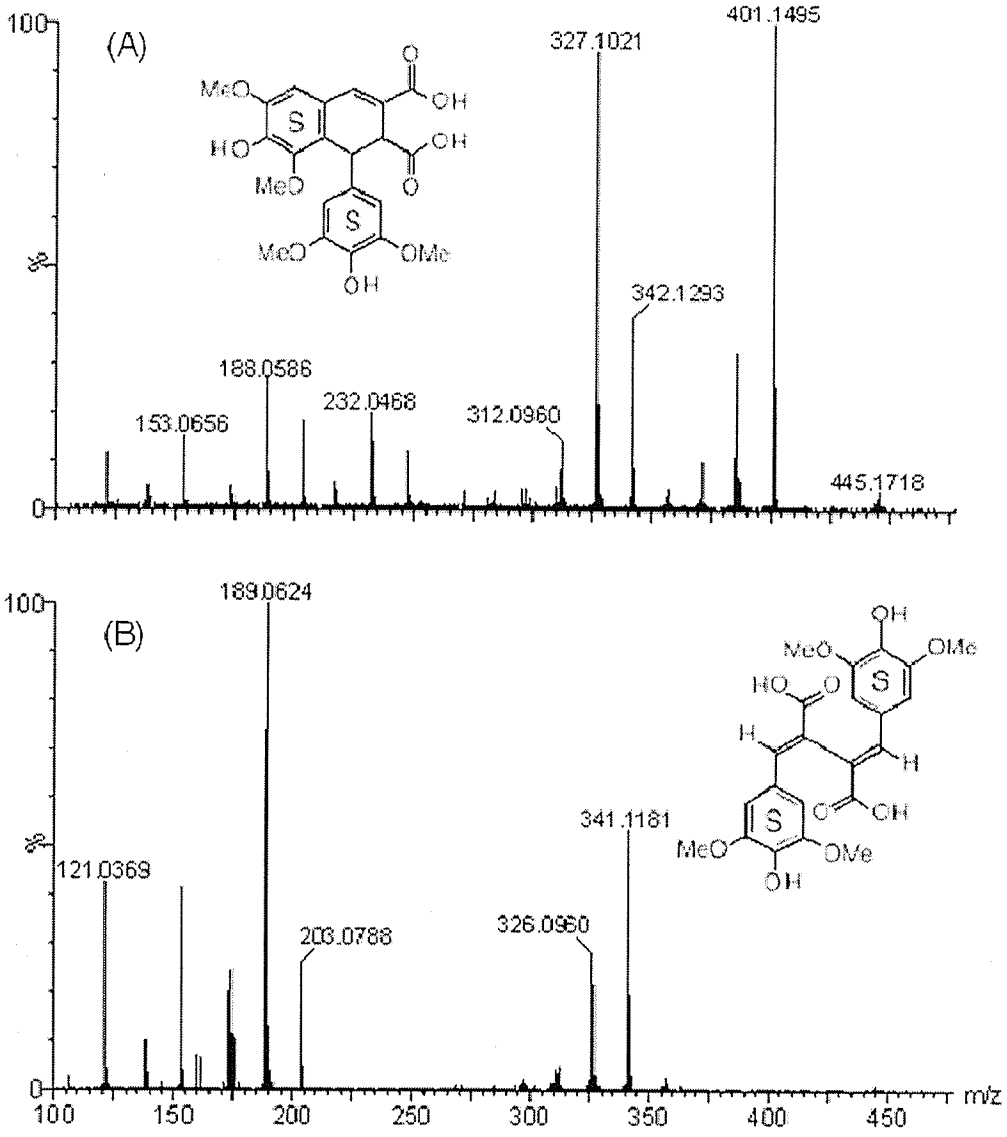


Figure 2.12. MS/MS spectra of disinapic acids (DSA) in wild rice (A) peak DS1 (8-8' aryltetralin form), and (B) peak DS2 (8-8').

To date, whether cyclic 8-8' coupled sinapate dehydrodimer (thomasidioic acid) is a natural product or an air oxidation artefact is still in disrepute. During alkaline-induced air oxidation, the conversion of sinapic acids to thomasidioic acids was observed by several authors (Rubino et al, 1995 & 1996; Charlton & Lee, 1997). The possible synthetic pathway (Cai et al, 1999) is illustrated in Figure 2.13. However, in our study, the noncyclic 8-8'-coupled form appeared at significant levels compared with the cyclic form, suggesting that the occurrence of thomasidioic acid is most likely natural.

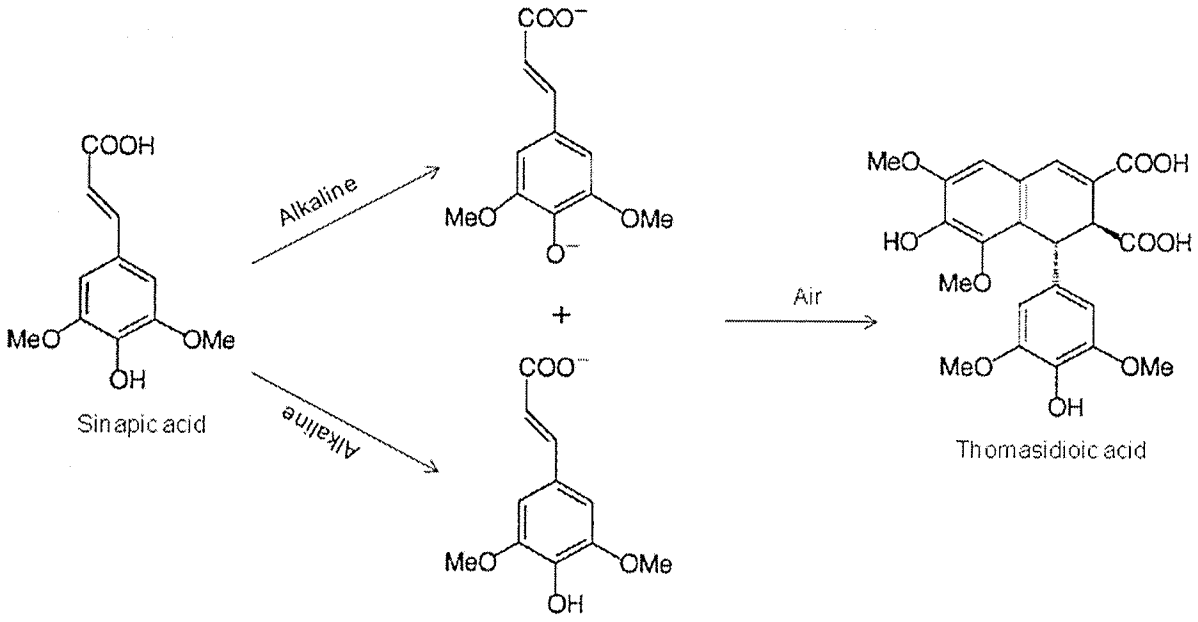


Figure 2.13. Conversion of sinapic acids to thomasidioic acid under alkaline condition with the presence of air.

In the soluble fractions, no phenolic dehydrodimers were detected. Although some peaks appeared between 50 min and 60 min (Figure 2.7), they were recognized as flavonoid glycosides as recently reported (Qiu, Liu, & Beta, 2009). A predominant peak (UK1) was unexpectedly present at 59.22 min with a typical UV absorption at 227 nm and 301 nm. As seen in Figure 2.14., the parent ion obtained from this peak was at m/z = 497, with only one major product ion at m/z = 237. By applying collision energy on the product ion (m/z = 237), a series of fragment ions were obtained. The characteristic mass losses observed from these fragments were 15 Da, 30 Da and 44 Da, which were probably due to the eliminating of $-CH_3$, $-OCH_3$ and –COOH group, respectively. The fragment ions at m/z = 193, and 163 suggested that UK1 may be a cinnamic acid derivative.

2.4. Conclusions

The methanol extracts of wild rice displayed up to 10 times higher antioxidant activity than white rice control by using DPPH photometric method and ORAC method, which signifies that more health benefits may be obtained by consuming wild rice than white rice. The processed sample, quick cooking wild rice and the mixed sample exhibited lower antioxidant activity compared with raw samples. After alkaline treatment, several monomeric phenolic acids were identified in both soluble and insoluble fractions with the most quantities of ferulic and sinapic acids. Phenolic dehydrodimers were only observed in the insoluble fractions. They mainly consisted of 8-O-4' coupled diferulic acids and 8-8' coupled disinapic acids. The TPC and DPPH radical scavenging activities of soluble and insoluble fractions suggested that phenolic acids

constitute a small portion of phenolic compounds and contribute to the antioxidant activity of wild rice.

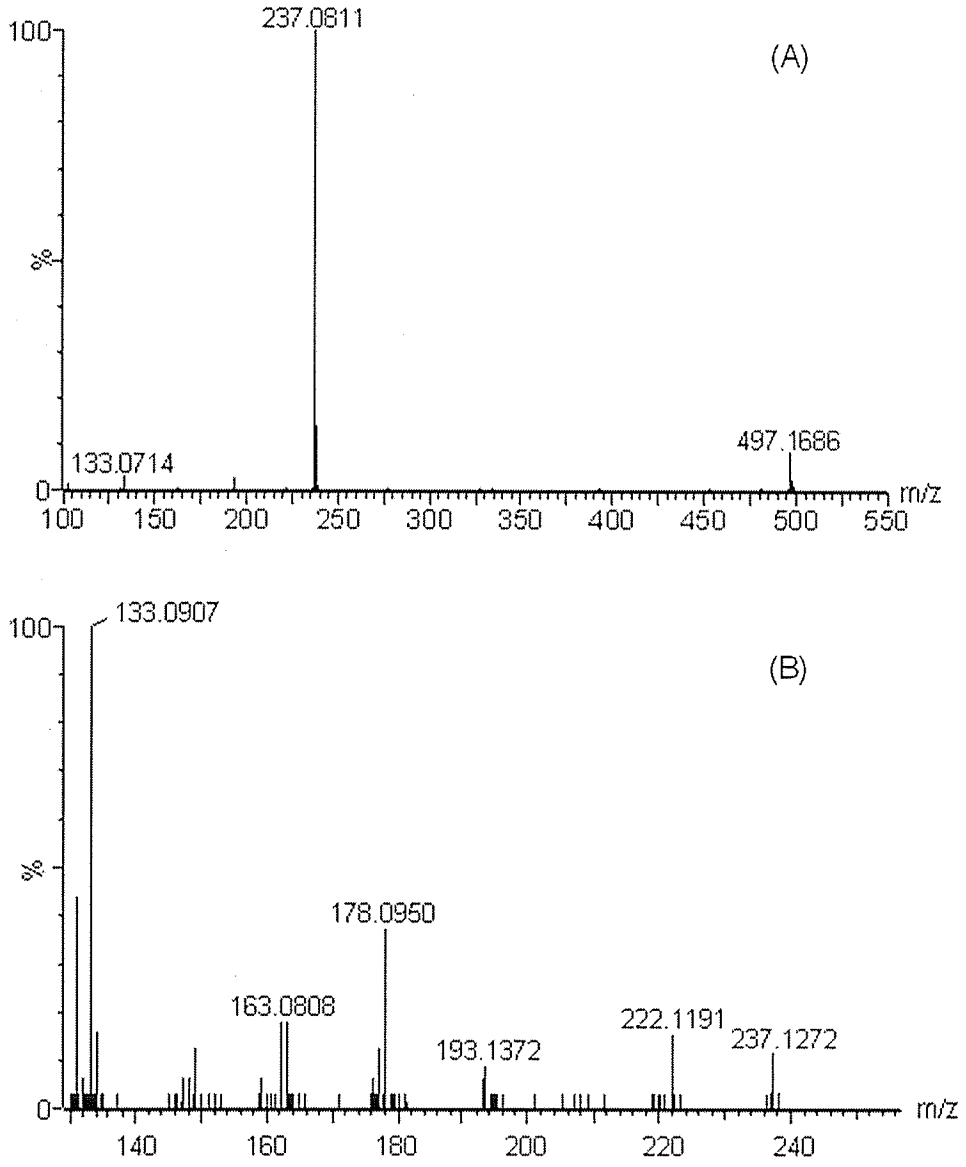


Figure 2.14. (A) MS/MS spectra of ions at *m/z*=497; (B) fragmentation pattern of ions at *m/z*=237.

CHAP TER 3: Antioxidant activity of commercial wild rice and identification of flavonoid compounds in active fractions

ABSTRACT

The health benefits of whole grain consumption have been attributed to their content of complex carbohydrates, vitamins, minerals and other phytochemical constituents. Wild rice is a whole grain finding applications in gourmet foods due to its nutritional value and unique taste. However, little is known about its antioxidant properties and phytochemical components. The objectives of this study were to evaluate the antioxidant properties of wild rice. Eleven commercial wild rice samples (raw, mixed, and processed) were extracted with acetone and fractionated using a Sephadex LH-20 column. 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH·) scavenging activity, oxygen radical absorbance capacity (ORAC) and total phenolic content were evaluated to determine the antioxidant properties of wild rice. The antioxidant activity of wild rice was found to be 30 times greater than that of the control white rice. Significant differences ($p<0.05$) in antioxidant activities were found among raw, mixed and processed samples. For raw samples, DPPH· radical scavenging activities and ORAC values ranged from 611 to 917 μmol Trolox Equivalent (TE) /100 g, and from 4069 to 6064 μmol TE/100 g, respectively. For mixed and processed wild rice, DPPH· radical scavenging activities were 373 and 441 μmol TE/100 g, respectively. The corresponding ORAC values were 2284 and 2557 μmol TE/100 g. Total phenolic content (TPC) of raw wild rice varied from 2472 to 4072 mg of ferulic acid equivalent (FAE)/kg, higher than that of the mixed sample (1460 mg FAE/kg) and processed sample (2076 mg FAE/kg). TPC was highly correlated with total antioxidant activity of wild rice ($r=0.92$). By

applying tandem mass spectrometric techniques, the antioxidants identified in wild rice were flavonoid glycosides (di-glucosyl apigenin, glucosyl-arabinosyl apigenin, and di-arabinosyl apigenin) in factions 2 and 3 and flavan-3-ols (catechin, epicatechin and oligomeric procyanidin) in fractions 4 and 5.

3.1. Introduction

Wild rice (*Zizaniae. palustris* & *Zizaniae. aquatica*) indigenous to the northern United States and southern Canada, is historically consumed by Native Americans as a staple food (Moyle, 1944; Lorenz, 1981a; Lorenz, 1981b; Steeves, 1952). In the late 20th century, wild rice was commercially cultivated to meet the increased demand (Oelke & Boedicker, 1991). Due to its unique flavour, wild rice is currently used in a wide range of gourmet food products including soups, salads, and desserts (Oelke et al, 1997). Most wild rice is sold in the raw form, but mixed wild rice and processed wild rice including quick cooking wild rice are also available on market.

With regards to the nutritional components, wild rice is high in protein and starch (Becker & Lorenz, 1981; Grava & Raisanen, 1978; Lorenz, 1981b; Wang et al, 1978), and low in fat (Anderson, 1976; Wang et al, 1978). In 2006, wild rice was recognized as a whole grain by the U.S. Food and Drug Administration (FDA), an agency of the United States Department of Health and Human Services. It is universally accepted that regular consumption of whole grains is beneficial in human health, resulting in reduced incidence of chronic diseases (Anderson et al, 2000). Phytochemicals have been suggested to be the key contributors to these health benefits due to their antioxidant properties (Liu, 2007). The antioxidant properties of wild rice have

received limited attention in the literature. Few studies have focused on the role of wild rice in lipid peroxidation. When incorporated into meat products, crude or cooked wild rice was able to retard lipid oxidation (Johnson et al, 1996; Minerich et al, 1991; Rivera et al, 1996). Methanol extracts of wild rice grain and wild rice hull also enhanced the stability of ground beef implying they can be effective antioxidants (Asamarai et al, 1996; Wu, 1994). However, the specific antioxidants in wild rice have not been well studied with the exception of phytic acids (Becker & Lorenz, 1981; Wu, 1994).

The primary aim of this study was to evaluate the antioxidant properties of commercial wild rice and identify specific compounds responsible for antioxidant activity. A secondary objective of the investigation was to compare the antioxidant properties of raw, mixed and processed wild rice samples.

3.2. Materials and methods

3.2.1. Samples

The samples used in this study were described in previous chapter. Each sample was ground into fine powder (< 0.5 mm) using a cyclone mill (Model 3010-018, Udy Corporation, Fort Collins, CO, USA) and stored at -20 ° C before extraction.

3.2.2. Chemicals

Folin-Ciocalteau reagent, DPPH, and ferulic acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Procyanidin standards catechin, epicatechin, B1 and B2 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). HPLC grade acetone and methanol were used in the extraction and fractionation. MS grade water, acetonitrile and acetic acid were used in LC-MS analysis. All the HPLC grade and MS grade solvents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

3.2.3. Extraction

Ground rice (2.0 g) was added 40 mL of acetone: water: acetic acid (70: 29: 1, v/v/v) prior to sonication for 1 h at room temperature. The mixture was centrifuged at 5000 rpm (Model 2C5C, MANDEL, Guelph, Ontario) and 20 °C for 20 min. The supernatant was removed and used as crude extract to determine the antioxidant activity and total phenolic content. The crude extract was stored at -20 °C for further fractionation.

3.2.4. Fractionation

To detect procyanidins in wild rice, crude extracts was further purified by fractionation to avoid the contamination of sugars and other impurities. The fractionation method previously described by Gu et al. (2002) was used with some modifications.

Sample preparation: 3×100 mL of hexanes was added into the crude extract to remove lipids. The organic solvent was evaporated under vacuum at 35 °C. The residue was redissolved

with 5 mL of 50% methanol to get concentrated extract. Column preparation: Sephadex LH-20 was hydrated with 20% methanol for 2 h, and then manually packed into a glass column (50×1.5 cm). The Sephadex LH-20 column was conditioned using 250 mL of 20% methanol.

After loading the concentrated extract (5 ml), the column was eluted with 100 mL of 20% methanol, 150 mL of 60% methanol, and 100 mL of 70% acetone in that sequence. The eluents were collected separately. The 20% methanol eluent was collected as fraction 1 (F1). The 50 ml eluents of 60% methanol gave three fractions F2, F3 and F4. Lastly, the 70% acetone eluent produced F5. Each fraction was dried by a rotary evaporator (Model RE-51, Yamato Scientific America Inc., Santa Clara, CA) at 35 °C and then redissolved in 2 mL of 100% methanol. The concentrated fractions were kept at -20 °C for further analysis.

3.2.4. Measurement of total phenolic content

The total phenolic content (TPC) of crude extracts was evaluated by Folin-Ciocalteau method (Singleton & Rossi, 1965) with few modifications. Briefly, 200 μL of the appropriate dilutions of crude extracts were reacted with 1.8 mL of 10-fold diluted Folin-Ciocalteau reagent which was freshly made. The mixture was then neutralized with 1.8 mL of sodium carbonate (60 g/L). The absorbance was measured at 725 nm after 90 min of reaction at room temperature. Ferulic acid was used as the standard. Results were expressed as mg of ferulic acid equiv (FAE) per kg of rice (dry weight basis).

3.2.5. Determination of DPPH radical scavenging activity

This assay was based on the method of Brand-William (1995) as modified by Li et al. (2005). Briefly, a 200 μL of crude extract (or fraction) was added to 3.8 mL of 60 μM DPPH radical solution which was freshly made. After 60 min of incubation at room temperature, the absorbance at 515 nm was measured. DPPH free radical scavenging activities of crude extracts were expressed as μmol of Trolox Equivalents (TE) per 100 g of rice (dry weight basis) using a standard curve of trolox.

3.2.6. Evaluation of oxygen radical absorbance capacity

The oxygen radical absorbance capacity (ORAC) assay was based on the method described by Huang et al. (2004) and modified by Li et al. (2007). A Precision 2000 automated microplate pipetting system (BIO-TEK Instruments, Inc., Winooski, VT) was used for plate-to-plate transfer of solutions. An FL×800 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT) controlled by software KC4 3.0 (version 29) was used with fluorescence filters for an excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm.

First, 120 μL of fluorescence working solution was automatically transferred to a 96-well flat bottom polystyrene microplate (Corning Incorporated, Corning, NY, USA) and used as the substrate. Then 20 μL each of buffer solution (blank), Trolox (standard control), appropriately diluted samples and catechin (sample control) were added to the designated wells, respectively. After 20 min incubation at 37 °C, 60 μL of freshly made AAPH solution was added to each well to generate a peroxyl radical. The total reaction time was 50 min. The fluorescence of the reaction mixture was recorded every one minute.

The area under the fluorescence decay curve (AUC) was calculated according to the following equation:

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

where f_0 = initial fluorescence reading at 0 min and f_i = fluorescence reading at time i min. Final ORAC values were calculated as follows and expressed as μmol TE/100 g of rice (dry weight basis):

$$ORAC\ \ value = \left[\left(AUC_{sample} - AUC_{blank}\right)/\left(AUCtrolox - AUC_{blank}\right)\right] \times dilution\ \ factor$$

3.2.7. HPLC-MS/MS analysis

An HPLC (Waters 2695) equipped with a photodiode array detector (DAD) (Waters 996) and autosampler (717 plus, Waters) coupled with a quadrupole time-of-flight mass spectrometer (Q-TOF MS) (Micromass, Waters Corporation, Milford, MA) was employed for HPLC and mass spectometric analyses (LC-MS/MS). A 150 mm × 4.6 mm, 5 μm RP 18 column (Gemini, Phenomenex, USA) was used for separation. During the LC-MS analysis, 10 μl of sample was loaded and injected by an autosampler, and eluted through the column with gradient mobile phase consisting of A (water containing 0.1% acetic acid) and B (acetonitrile containing 0.1% acetic acid) with the flow rate of 0.5 ml/min, prior to introduction into the Q-TOF MS. A 50 min-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. Full mass spectra were recorded in negative mode by using the capillary voltage of 1.2 kV and cone voltage of 45 V. The flow rate of desolvation gas (N_2) and cone gas (He) were 900 L/h and 50 L/h, respectively. The desolvation temperature and the source temperature were set at 350°C and 150°C, respectively. The MS/MS spectra were acquired by using collision energy of 30 V.

All the samples were extracted and analyzed in triplicate.

3.2.8. Statistical analysis

The results were reported as mean ± standard deviation (SD). Data were analyzed by a one-way analysis of variance (ANOVA) test using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). Least significant differences (LSD) at $p< 0.05$ were tested to assess significant differences in antioxidant properties among samples.

3.3. Results and Discussion

3.3.1. Total phenolic content (TPC) in crude acetone extracts

Phenolic compounds are considered the major antioxidants occurring in fruits, vegetables, and cereals; therefore, the measurement of total phenolic content (TPC) tends to be important when assessing their contribution to antioxidant activity. Table 3.1 shows the TPC of crude, aqueous acetone extracts from white rice (control) and wild rice.

Table 3.1. Total phenolic content (TPC) in acetone extracts from white rice control and wild rice[a,c]

Class	No.	Sample	TPC (FAE[b] mg/ kg)
Control	WR	White rice control	279 ± 13i
Mixed	3M	3 blend mix	1460 ± 29h
Processed	QC	Quick cooking	2076 ± 43g
Raw	AB	A black	3575 ± 34c
	BB	B black	3342 ± 29d
	CS	C scarified	3796 ± 43b
	CL	Canadian lake	2472 ± 39f
	HH	Hand harvested	2498 ± 26f
	MC	Minnesota cultivated	3376 ± 22d
	M	Manomin	4072 ± 113a
	LS	Large size	2760 ± 68e
	SS	Small size	2743 ±32e

[a] Mean ± standard deviation (n=9).

[b] Ferulic acid equivalent.

[c] Least significant difference level of probability ($p<0.05$). Sample means with the same letter in the same column are not significantly different.

Raw wild rice (range 2472 - 4072 mg/kg) contained 10 to 15 times more TPC than white rice (279mg/kg). The TPC of mixed wild rice ranked between that of white rice and raw wild rice due to the dilution of the endogenous phenolics of wild rice by white rice and white basmati. Significant differences in TPC were found between raw and processed wild rice samples. Manomin and C scarified had the highest TPC among the raw samples; however, quick cooking

wild rice displayed significantly lower TPC (2076 mg/kg) than uncooked ones. Thus the process of producing the quick cooking product substantially lowers the phenolic levels found in wild rice. The general procedure for production of quick cooking wild rice involves soaking, cooking, and drying, conditions that results in loss of phytochemicals due to leaching in the liquid used or destruction and/or transformation of chemical structures present in raw grain (Dewanto, Wu, & Liu, 2002; Li, Pickard, & Beta, 2007). The loss of water-soluble and free antioxidants during rice cooking is also responsible for reduced levels of phenolics (Finocchiaro et al, 2007).

3.3.2. DPPH radical scavenging activity of crude acetone extracts

The DPPH· photometric method is widely employed for evaluating the free radical scavenging activity of hydrogen donating antioxidants in plant extracts (Brand-Williams, Cuvelier, & Berset, 1995). The DPPH· radical scavenging activities of crude acetone extracts presented in Figure 3.1 are expressed as μmol of trolox equivalents (TE) per 100 g of rice on dry weight basis.

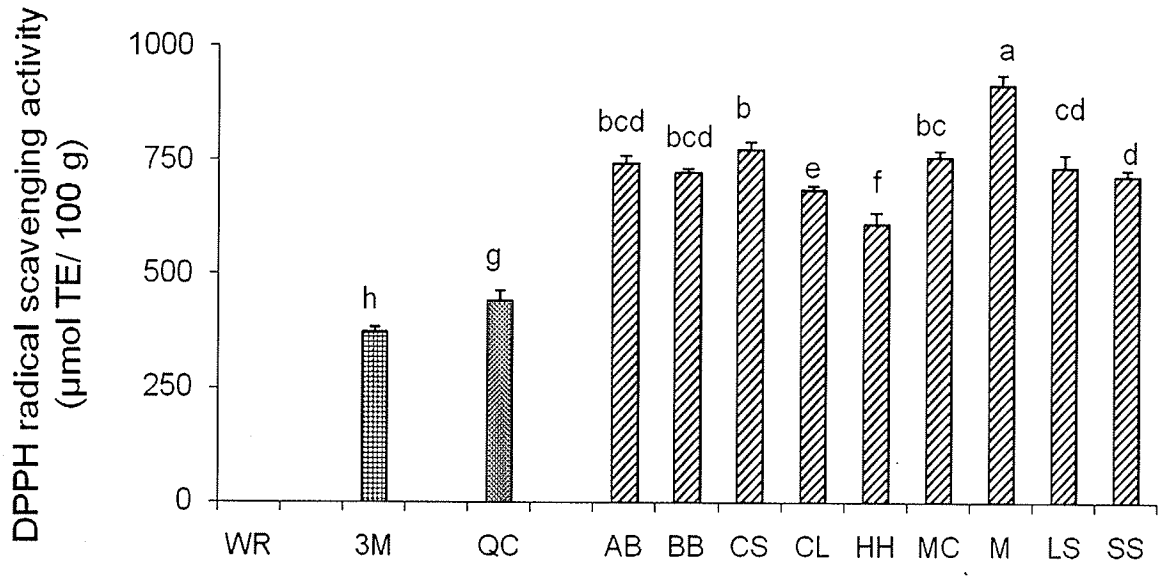


Figure 3.1. Free radical scavenging activity of acetone extracts from white rice and wild rice on DPPH radical (at 60 min). WR, white rice; 3M, 3 blend mix; QC, quick cooking; AB, A black; BB, B black; CS, C scarified ; CL, Canadian lake; HH, hand harvested; MC, Minnesota cultivated ; M, Manomin; LS, large size; SS, small size. The vertical bars represents the standard deviation (n=9). Columns marked by different letters are significantly different ($p < 0.05$).

Because of the very low levels, the DPPH· scavenging activity of the white rice control could not be estimated according to the trolox standard curve. The DPPH· scavenging activities of raw samples (717 to 917 μmol TE/100 g) were significantly higher ($p < 0.05$) than that of the mixed wild rice (373 μmol/100 g) . The highest and lowest values were observed in Manomin and hand harvested wild rice, respectively. The DPPH· scavenging activities were not significantly different between A and B black wild rice, as well as between large and small sized wild rice ($p>0.05$). The grade and the size of wild rice did not affect antioxidant activity among the samples examined. Significant differences in DPPH· scavenging activity were found between raw and processed samples. The average DPPH· scavenging activity of raw wild rice (741 μmol TE/ 100 g) was 40% higher than that of quick cooking wild rice. These results are consistent with the reduction in TPC observed in quick cooking wild rice.

3.3.3. Oxygen radical absorbance capacity of crude acetone extracts

In the oxygen radical absorbance capacity (ORAC) assay, antioxidant activity is determined by using fluorescein as the fluorescent probe and 2,2'-azobis(2-amidinopropane) dihydrochloride (APPH) as the peroxyl radical generator. Due to its biological relevance to *in vivo* antioxidant efficacy, it has been widely used to investigate the antioxidant activity of foods including fruits, vegetables, nuts, cereals and spices (Dávalos et al, 2004; Wu et al, 2004). Wu et al. (2004) reported that hydrophilic ORAC (H-ORAC) values are much higher than lipophilic ORAC (L-ORAC) values, contributing to 95% of the total antioxidant capacity for most food. Finocchiaro et al. (2007) also pointed out that the antioxidant capacity of red and white rice were

not due to the lipophilic compounds. Therefore, only H-ORAC assay was carried out in our study.

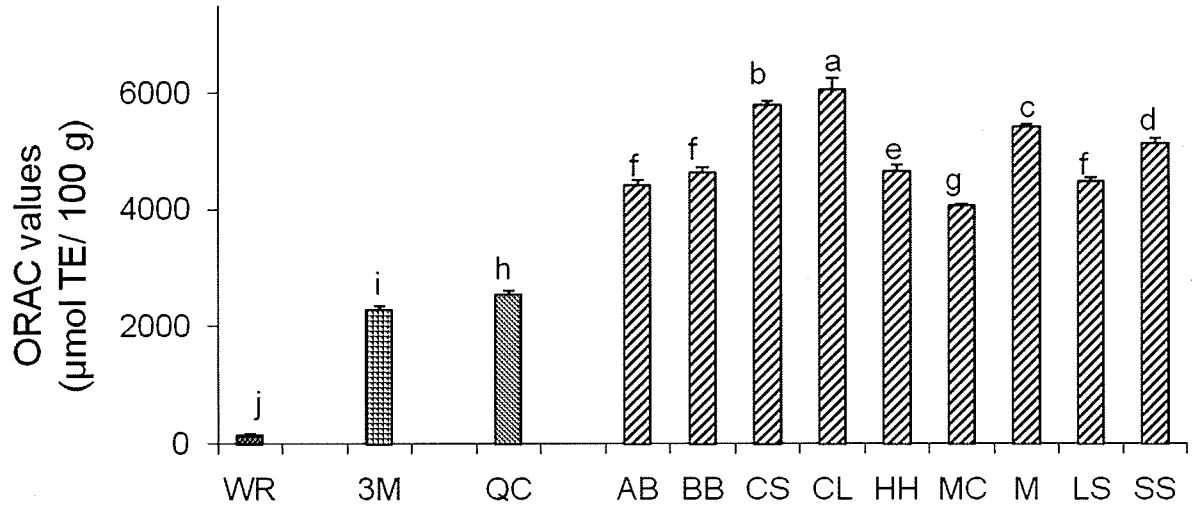


Figure 3.2. ORAC values of acetone extracts from white rice and wild rice. WR, white rice; 3M, 3 blend mix; QC, quick cooking; AB, A black; BB, B black; CS, C scarified ; CL, Canadian lake; HH, hand harvested; MC, Minnesota cultivated ; M, Manomin; LS, large size; SS, small size. The vertical bars represents the standard deviation (n=9). Columns marked by different letters are significantly different ($p < 0.05$).

H-ORAC values of white rice control and wild rice are shown in Figure 3.2, expressed as μmol TE per 100 g of rice on dry weight basis. For the control, the H-ORAC value was 153 μmol TE/100 g. Contrary to the control, raw wild rice had significantly high H-ORAC values ranging from 4069 to 6064 μmol TE/100 g for Canadian and Minnesota cultivated samples, respectively. High ORAC values were also observed in C scarified (5799 μmol TE/100 g) and Manomin (5421 μmol TE/100 g) wild rice. Although lower than raw wild rice samples (average 4970 μmol TE/100 g), the H-ORAC value of mixed wild rice (2284 μmol TE/100 g) was 15 times higher than that of the control white rice. Mixing white rice with wild rice can therefore improve the peroxyl radical scavenging activity of white rice. Consistent with DPPH scavenging activity results, processed wild rice had a significant lower value of H-ORAC than raw samples. The H-ORAC value of quick cooking wild rice was 2557 μmol TE/100 g, about half the average value of the raw samples.

Significant differences in antioxidant activities among some raw wild rice samples can be attributed to several complex factors including cultivar, growing environment, and harvesting conditions. According to Mitchell et al. (2007), organic food products have higher antioxidant activity than inorganic or conventional food products which may explain the higher antioxidant activity of Manomin wild rice than Minnesota cultivated wild rice. Manomin wild rice is organically grown at Wabigoon Lake by Ojibway aboriginal people in Northwestern Ontario, Canada, whereas Minnesota cultivated wild rice is grown in a man-made paddy instead of a natural lake or river. Environmental variations such as growing location and temperature also considerably affect the content of antioxidants and the antioxidant activity of cereals (Mpofu, Sapirstein, & Beta, 2006).

3.3.4. Antioxidant activity of fractions (F1 to F5)

The crude extract was fractionated with methanol and acetone on a Sephadex LH-20 column. The antioxidant activities of each fraction were evaluated by measurement of their DPPH scavenging ability and expressed as % DPPH· quenched at 60 min. The results are summarized in Table 3.2.

For raw samples, the DPPH· radical scavenging activities of ranged from 2.33% to 4.39% for F1 , 15.01% to 19.69% for F2, 12.61% to 16.80 for F3, 3.45% to 7.64 % for F4, and 9.66% to 11.97 % for F5. A decline in DPPH· radical scavenging activities was found in the following order: F2 > F3 > F5 > F4 > F1. The antioxidants were, therefore, more abundantly distributed in F2, F3 and F5 than in other fractions. As suggested by Gu et al. (2002), the compounds in the first fraction (F1) eluted with 20% methanol are mainly sugars; the compounds eluted with 60% methanol (F2, F3 and F4) are simple phenolic acids and other low molecular weight phenolics; and the 70% acetone eluent (F5) may consist of some polymerized phenolic acids and other high molecular weight polyphenol constituents. To identify and characterize the antioxidants in wild rice, the active fractions (F2-F5) were analyzed by LC-MS and MS/MS analysis.

Table 3.2. DPPH scavenging activity (%) of each fraction (F1 to F5) isolated from wild rice and eluted from Sephadex LH-20 column[a, b]

Class	Sample name	F1	F2	F3	F4	F5
Control	White rice	nd[c]	nd	nd	nd	Nd
Mixed	3 blend mix	1.50±0.04	11.32±0.82	9.49±0.50	5.02±0.21	7.75±0.41
Processed	Quick cooking	1.77±0.04	13.52±0.74	11.89±0.28	4.65±0.06	6.15±0.07
Raw	A black	3.70±0.18	17.99±0.18	16.58±0.24	5.65±0.30	9.66±0.41
	B black	2.33±0.09	15.86±0.21	14.10±0.66	3.45±0.11	10.87±0.56
	C scarified	4.39±0.20	17.01±0.75	15.87±0.59	7.64±0.08	11.56±0.39
	Canadian lake	3.91±0.07	15.32±0.48	14.95±0.37	5.55±0.32	9.69±0.25
	Hand harvested	2.47±0.23	15.59±0.27	12.61±0.80	4.47±0.07	10.40±0.44
	Minnesota cultivated	3.56±0.15	16.71±0.82	15.85±0.37	5.12±0.10	13.92±0.40
	Manomin	4.26±0.20	19.69±0.23	16.80±0.43	6.21±0.09	11.97±0.18
	Large size	3.28±0.18	16.85±0.40	13.93±0.77	4.85±0.4	11.51±0.32
	Small size	3.22±0.05	15.81±0.66	14.30±0.35	7.58±0.34	11.68±0.16

[a] The fractions used for analyzing the DPPH radical scavenging activity were obtained from once fractionation.
[b] Mean ± Standard deviation (n=3).
[c] Not detectable.

3.3.5. Identification of flavonoid glycosides in F2 and F3

Flavonoids have received considerable attention due to their antioxidant activity (Pietta, 2000). As the secondary metabolites in plants, in most cases, flavonoids are conjugated with sugars and generally occurring as flavonoid *O*- or *C*-glycosides (Wollenweber, 1993). The unconjugated flavonoids without a sugar moiety are called aglycones among which flavones, flavonols and flavanones are the most commonly encountered (Stobiecki, 2000). As reported by van Acker et al. (1996), antioxidant activity of flavonoids is related to their structural aspects with better scavenging activity being associated with the presence of the catechol moiety in ring B. Thus, HPLC-MS/MS was applied in our study to characterize flavonoids in wild rice and investigate their chemical structures.

Figure 3.3 (a) and (b) are the HPLC chromatograms (330 nm) of F2 and F3 eluted from Manomin wild rice acetone extract. The major peaks were numbered according to their elution time. As seen in Figure 3.3 (a), peak 2 (t_R = 25.58 min) and peak 3 (t_R = 28.57 min) together with a smaller peak (peak 1, t_R = 23.12 min) were the major peaks detected in F2. In comparison to F2, F3 contained more peaks with longer retention that abundantly occurred between 25 min and 32 min (Figure 3.3 (b)). Due to the similarity of the retention times (Figure 3.3 (d)), peak 2' (t_R = 25.67 min) and peak 3' (t_R = 28.68 min) observed in F3 presumably contained the same compounds as peak 2 and peak 3 in F2. The UV spectra of all the numbered peaks were characterized by two major absorption bands: I around λ_{max} 330 nm and II at λ_{max} 270 nm. As a representative, the UV spectrum of peak 3 is shown in Figure 3.3 (c).

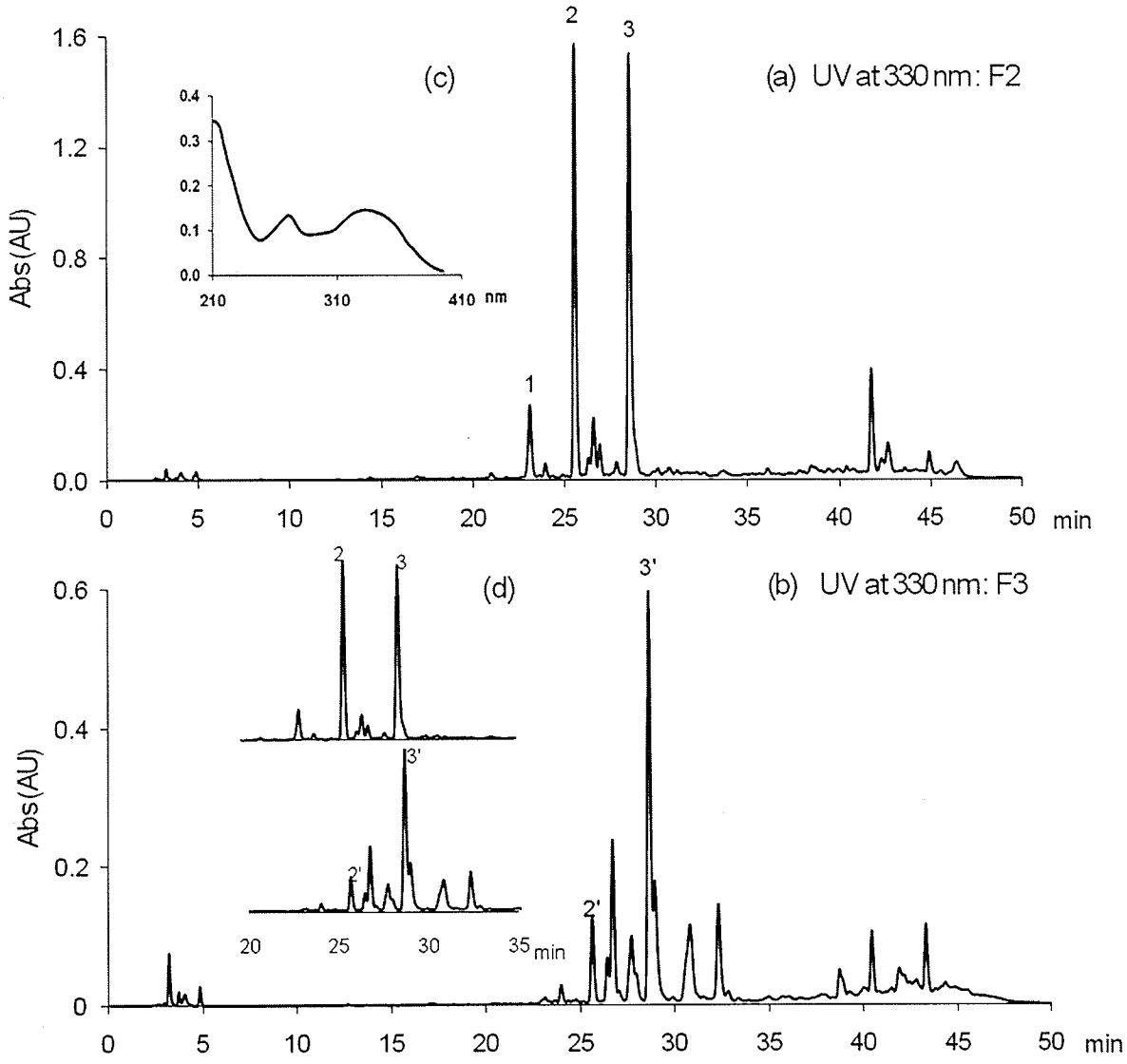


Figure 3.3. (a) Full LC chromatograms (0-50 min) recorded at 330 nm of fraction 2 (F2) isolated from Manomin wild rice; (b) full LC chromatograms (0-50 min) recorded at 330 nm of fraction 3 (F3) isolated from Manomin wild rice; (c) full UV spectra of peak 3 ; (d) highlights of LC chromatograms (20-35 min) for F2 and F3.

In mass analysis, negative ion mode was selected because it provided extensive structural information via collision-induced dissociation. In order to facilitate discussion on mass fragmentations, the nomenclature of product ions introduced by Domon and Costello (1988) was employed here with slight modifications. The letter A represents the aglycone. Fragment ions from deprotonated molecules containing the aglycone part are denoted X^- and Y^-. The X^- ions are formed by cleavage within the glycosidic rings and usually labeled ${}^{k,l}X_j^-$. The subscript *j* represents the number of the interglycosidic bond counting from the aglycone with the glycosidic bond linking to the aglycone being numbered 0. The superscripts *k* and *l* indicate the ring bonds that have been broken in the sugar residues. In the case of diglycosides, ${}^{(k,l),(k,l)'}X_j^-$ is employed for the ions formed by simultaneous losses from both sugars. The Y^- ions are the characteristic ions for *O*-glycosides produced by cleavage at glycosidic *O*-linkages which leads to the complete loss of one or more sugar moieties. In the case of diglycosides, Y_1^- is formed after loss of one sugar residue, and Y_0^- corresponds to the loss of two sugar residues.

The mass spectra of peak 1, 2, and 3 are displayed in Figure 3.4. It is clear to see that the parent ions $[M-H]^-$ of these three peaks were at *m/z* = 593, 563, and 533, respectively. The ions at *m/z* = 1187, 1127, and 1067 were the clusters of the parent ions, which were formed during the dissovation. Figure 3.5 shows the typical MS/MS spectra of peak 1, 2 and 3. The relative ion intensity of product ions are shown in Table 3.3.

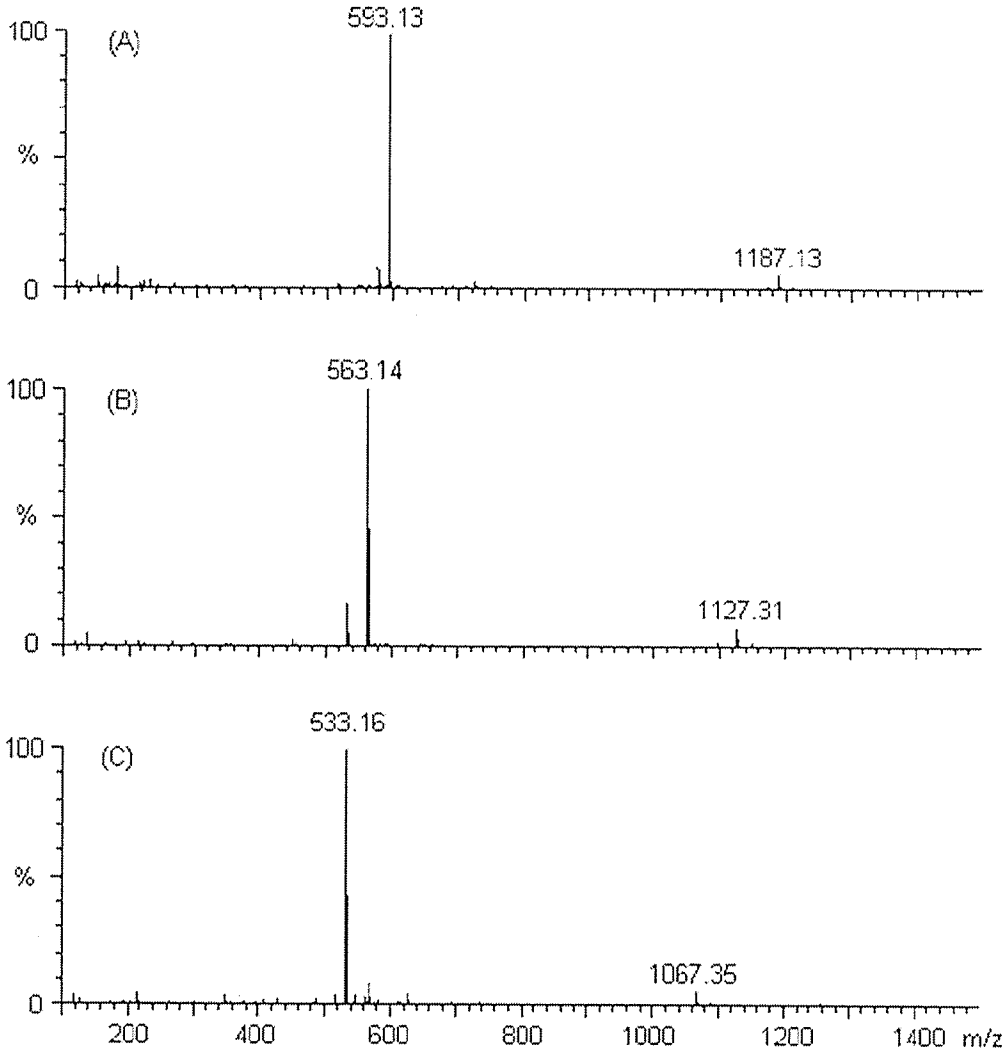


Figure 3.4. The ESI-QTOF mass spectra in negative ion mode for (A) peak 1 (22.8-23.3 min), (B) peak 2 (25.2-25.8 min) and (C) peak 3 (28.2-29.0 min) of fraction 2 (F2) isolated from wild rice.

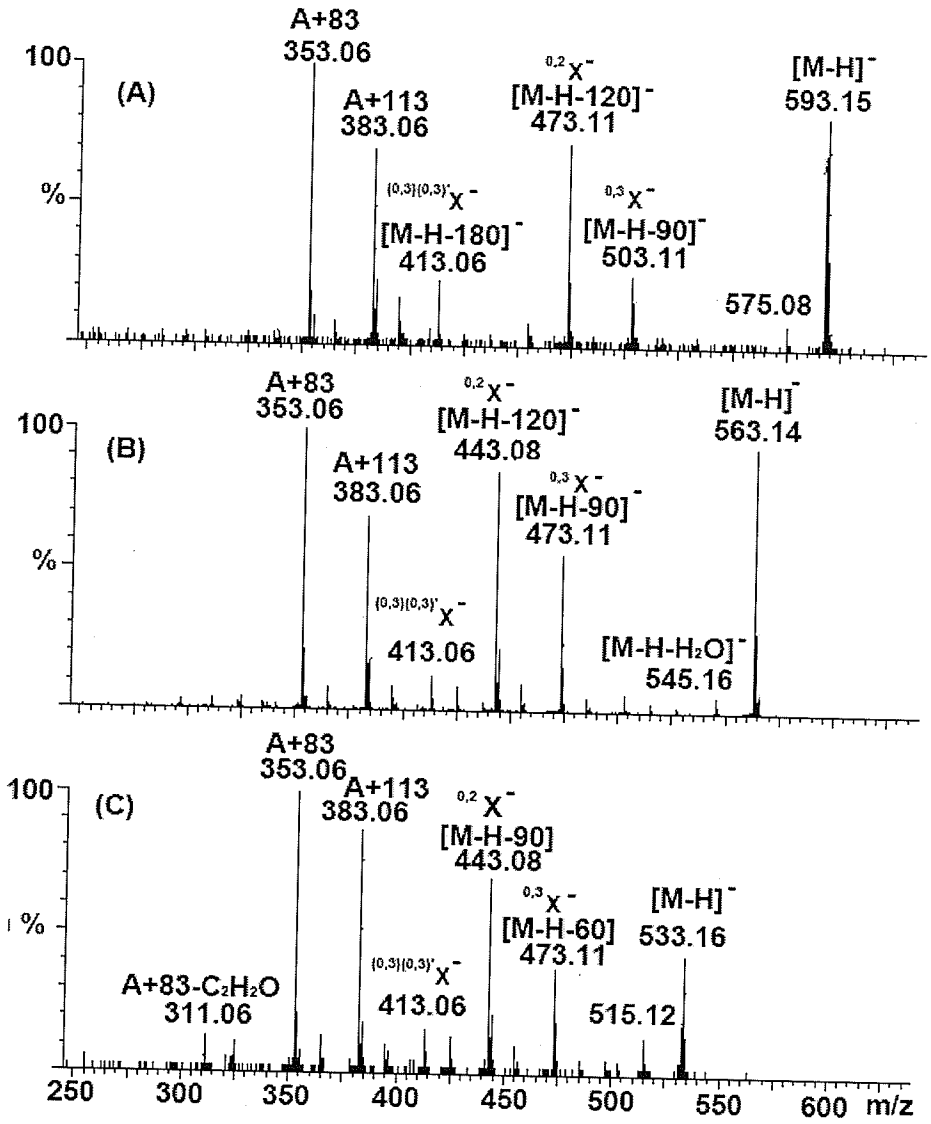


Figure 3.5. MS/MS spectra in negative ion mode for (A) peak 1, (B) peak 2, and (C) peak 3 of fraction 2 (F2) isolated from wild rice.

Table 3.3. Retention time, maximum UV absorption, deprotonated molecular mass, fragmentation pattern of ion loss and relative intensity of product ions of peak 1, 2, and 3 of fraction 2 (F2) isolated from wild rice.

Peak	t_R	λ_{max}	$[M-H]^-$	Relative intensity of product ions (%)					
no	(min)	(nm)	*m/z*	$-H_2O$	−60	−90	−120	A+83[a]	A+113
1	23.13	270, 334	593	5	0	28	83	100	73
2	25.58	271, 328	563	3	4	55	85	100	70
3	28.57	270, 331	533	2	38	71	0	100	85

[a] Base peak

Peak 1 (t_R = 23.12 min): MS, $[M-H]^-$ 593; MS/MS, $[M-H-18]^-$ 575, $[M-H-90]^-$ 503, $[M-H-120]^-$ 473, $[M-H-210]^-$ 383, $[M-H-240]^-$ 353, $[M-H-254]^-$ 339. The presence of ions at *m/z* 353 (A+83) and 383 (A+113) indicated that the algycone was apigenin (4',5,7-trihydroxyflavone) (Ferreres et al, 2003). The ions at *m/z* 503 and 473 corresponding to the loss of 90 Da and 120 Da from the deprotonated molecule $[M-H]^-$ were formed by cross-ring cleavages in a hexose residue, thus labeled $^{0,3}X_0^-$ and $^{0,2}X_0^-$ in Figure 3.5 (A). The ions at *m/z* 413 denoted $^{(0,3)(0,3)'}X^-$ revealed the simultaneous losses of 90 Da from both hexose residues. The water loss was revealed by the detection of the ions at *m/z* 575. The above characteristic losses (18, 90, and 120 Da) which are usually considered diagnostic for *C*-glycosides indicated that peak 1 was one of them. In contrast to *C*-glycosides, the typical sugar losses for *O*-glycosides are 162 (hexose), 146 (deoxyhexose) and 132 (pentose), and there is no water loss detected. The high intensity of $[M-H]^-$ (relative intensity> 85%) and the absence of Y_0^- ($[M-H-162-162]^-$

m/z=269) and Y_1^- ([M−H−162]$^-$ *m/z*=431) also suggested a *C*-glycosylation. To date, *C*-glycosylation is only found at *C*-6 and *C*-8 positions of the flavonoid aglycone (Cuyckens & Claeys, 2004). Therefore, peak 1 was designated as 6,8-di-*C*-hexosyl apigenin (M_r=594), comprising apigenin (270) and two hexoses (162+162). According to the previous reports on the product ions from [M−H]$^-$ 593 or [M+H]$^+$ 595 (Bakhitiar et al, 1994; Bouillant et al, 1979; Ferreres et al, 2003; Grayer et al, 2000; Lin, Chen, & Harnly, 2008), the most probable flavonoid glycoside corresponding to peak 1 is 6,8-di-*C*-glucosyl apigenin (vicenin-2).

Peak 2 (t_R = 25.58 min): MS, [M−H]$^-$ 563; MS/MS, [M−H−18]$^-$ 545, [M−H−90]$^-$ 473, [M−H−120]$^-$ 443, [M−H−180]$^-$ 383, [M−H−210]$^-$ 353, [M−H−238]$^-$ 325. The same alycone moiety (apigenin) was assumed for peak 2 due to the occurrence of A+83 (*m/z* 353) and A+113 (m/z 383) (Figure 3.5 (B)). The ions at *m/z* 473 and 443 exhibited the characteristic sugar losses of *C*-glycosides (90 Da and 120 Da). The ions at *m/z* 413 were likely formed after loss of 60 Da from pentose and further loss of 90 Da from hexose. The ions at *m/z* 545 formed by the loss of water further pointed to the *C*-glycosylation. Thus, peak 2 was assigned as hexosyl-pentosyl apigenin (M_r= 564, apigenin 270+ hexose 162 + pentose 132). 6-*C*-glucosyl-8-*C*-arabinosyl apigenin and 6-*C*-arabinosyl-8-*C*-glucosyl apigenin are in the greatest possibility. As reported by Ferreres et al. (2003), 6-*C*-glucosyl-8-*C*-arabinosyl apigenin gave rise to [M−H−120]$^-$, while 6-*C*-arabinosyl-8-*C*-glucosyl apigenin was pronounced for [M−H−90]$^-$. Thus, the former is preferred here since the higher relative intensity of [M−H−120]$^-$ was observed in peak 2 (Table 3.3).

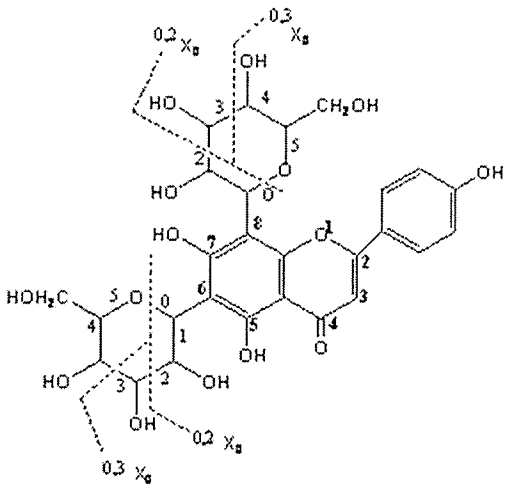
Peak 3 (t_R = 28.57 min): MS, [M−H]$^-$ 533; MS/MS, [M−H−18]$^-$ 515, MS/MS, [M−H−60]$^-$ 473, [M−H−90]$^-$ 443, [M−H−150]$^-$ 383, [M−H−180]$^-$ 353, [M−H−222]$^-$ 311. As

seen in Table 3, the ions at *m/z* 383 (A+113) were still of the highest intensity as the base peak with the second most abundant ions observed in A+83 at *m/z* 353 (Figure 3.5 (C)), indicating the aglycone of apigenin. However, the relative intensity of the precursor ion $[M-H]^-$ went down when comparing with peak 1 and peak 2. A water loss was observed by the presence of the ions at *m/z* 515.12. The most distinguished loss observed in peak 3 from previous two peaks was the abundant loss of 60 Da originating from pentoses. As reported by previous authors (Cuyckens et al, 2000; Stobiecki, 2000), the loss of 60 Da and 90 Da were the characteristic sugar losses for *C*-glycosides, formed by cross-cleavages within pentose residues. Thus, peak 3 was designated as *C*-dipentosyl apigenin containing apigenin and two pentose residues.

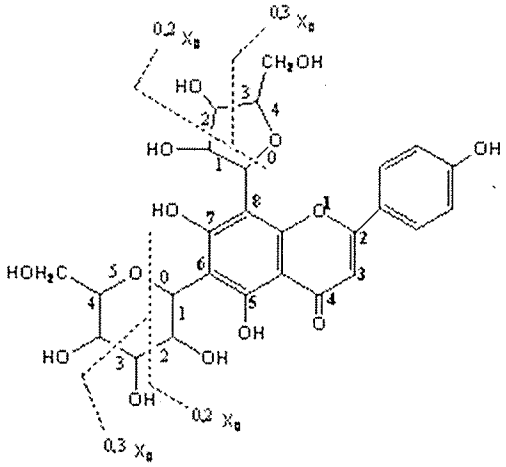
The general terms hexose and pentose were used for sugar moieties due to the difficulty in detection of stereochemical structures of carbohydrate residues. Glucose is the most commonly encountered sugar; rhamnose, xylose and arabinose are less common; while mannose and fructose are rare (Cuyckens & Claeys, 2004). The glycosylation positions are also challenging to determine or identify. In the case of *C*-glycosides, 6-*C* and 8-*C* are the only two positions and the differentiation can be made by high energy collision-induced dissociation (CID) according to the abundance of water loss (preferable at 6-*C* position). Figure 3.6 is used to illustrate the major fragmentation pathways in peak 1, 2 and 3, when they are designated as 6,8-di-*C*-glucosyl apigenin, 6-*C*-glucosyl-8-*C*-arabinosyl apigenin, and 6,8-*C*-di-arabinosyl apigenin, respectively.

Peak 2' and 3'observed in F3 giving the same MS and MS/MS spectra as peak 2 and 3 confirmed the previous assumption that they comprised the same compounds. In addition to peak 2' and 3', the other peaks detected in F3 with retention times between 20 min to 35 min were

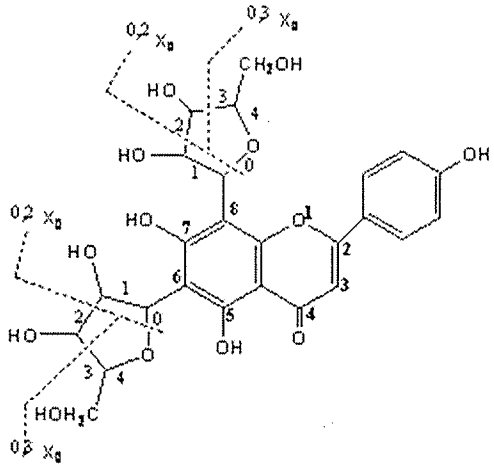
found as the isomers of peak 1, 2 and 3, when plotting the extraction ion chromatograms of ions at *m/z* =593, 563, and 533 (Figure 3.7). Thus, flavone glycosides were likely the predominant antioxidant compounds detected in F2 and F3.



Peak 1: 6, 8-di-C-glucosyl apigenin



Peak 2: 6-C-glucosyl-8-C-arabinosyl apigenin



Peak3: 6, 8-di-C-arabinosyl apigenin

Figure 3.6. The fragmentation pathways assumed in peak 1 (6, 8-di-C-glucosyl apigenin), 2 (6-C-glucosyl-8-C-arabinosyl apigenin) and 3 (6, 8-di-*C*-arabinosyl apigenin) of fraction 2 (F2) isolated from wild rice.

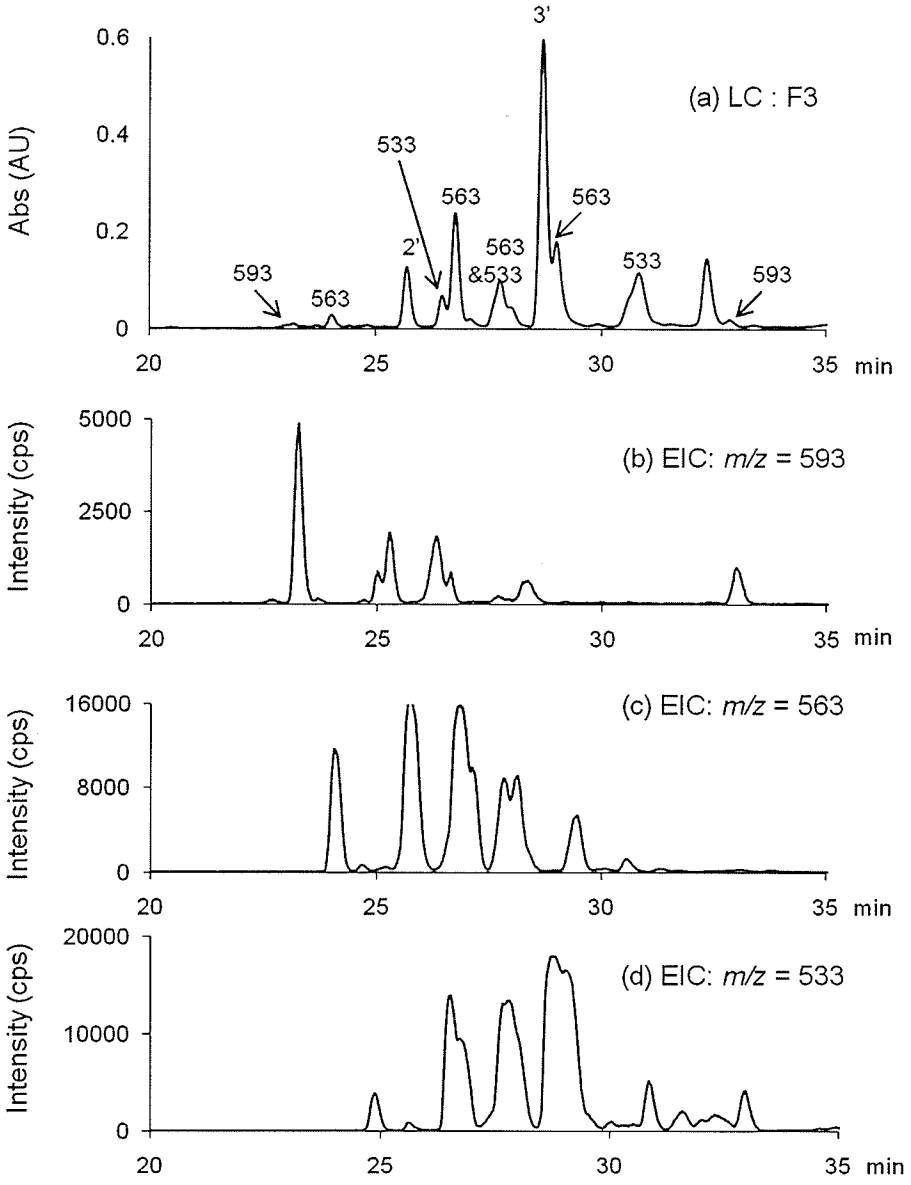


Figure 3.7. (a) Highlights of LC chromatograms (20-35 min) for F3 from Manomin wild rice recorded at 330 nm; Extracted ion chromatogram (EIC) of ions at (b) *m/z* = 593; (c) *m/z* = 563; (d) *m/z* =533.

3.3.6. Identification of monomeric and oligomeric flavan-3-ols in F4 and F5

Flavan-3-ols, namely catechins and procyanidins are a subclass of flavonoids commonly found in plants and have been demonstrated to exhibit radical scavenging activity (Pietta, 2000; Plumb et al, 1998). Cereal procyanidins have been well characterized on barley, wheat, and sorghum (Brandon et al, 1982; Gujer et al, 1986; McCallum et al, 1990; McMurrough et al, 1983; Olschläger et al, 2008). However, only limited literature can be found on rice in general. Red rice only has been reported to contain oligomeric procyanidins with degree of polymerization (DP) ranging from 1 to 8 (Finocchiaro et al, 2007). To complement this knowledge, procyanidins in wild rice were investigated in our study. In agreement with Taylor et al (2003), the fractionation of crude acetone extracts prior to LC-MS analysis greatly improved the separation and detection of procyanidins, especially for those with low molecular weight. Nevertheless, the oligomers with DP $\geq$ 6 and the polymeric procyanidins were not detected in wild rice extracts by LC-MS.

Figure 3.8 (a) is the HPLC chromatogram (at 280 nm) of the standards consisting of (+)-catechin, (-)-epicatechin, B1, and B2. Figure 3.8 (b) shows the LC chromatogram of F4 isolated from Manomin wild rice. By comparison with retention times of standards, the peaks at 19.22 min (peak 4) and at 23.50 min (peak 5) were respectively designed as (+)-catechin and (-)-epicatechin. The UV spectra of these two peaks recorded by DAD showed a maximum absorption at 279.68 nm, the same as the standards. To further confirm the identification, analysis were conducted using Q-TOF MS and MS/MS. The total ion chromatogram (TIC) of F4 in negative ion mode is shown in Figure 3.8 (c). By plotting the extracted ion chromatogram (EIC) of a single ion at m/z = 289 from Figure 3.8 (c), it can been seen that peak 4 and peak 5 originated from the ions at m/z 289. Two peaks were observed in Figure 8(d), the first and most

abundant one was detected at 19.22 min corresponding to (+)-catechin while the second and smaller peak was present at 23.49 min corresponding to (-)-epicatechin. The intensity of (+)-catechin was found 4 times higher than (-)-epicatechin. To further confirm the identification of catechin monomers in F4, the fragmentation patterns of peak 4 and peak 5 were recorded by tandem mass spectrometry and characterized by the abundant ions $[M-H-44]^-$ at *m/z* 245, which were in concordance with the molecular masses of catechin and epicatechin standards. Thus it became evident that peak 4 was (+)-catechin and peak 5 was (-)-epicatechin. As suggested by Flamini (2003), these fragment ions were formed by the loss of a $-CH_2-CHOH-$ group from the precursor molecular ion $[M-H]^-$ at *m/z* 289.

Figure 3.9 (a) is the UV chromatogram (at 280 nm) of F5 for Manomin wild rice. The extracted ion chromatograms of deprotonated molecular ions $[M-H]^-$ at *m/z*=577, 865, 1153, and 1441 (Figure 3.9 (b), (c), (d), and (e)) indicated the presence of dimer, trimer, tetramer and pentamer of procyanidins, respectively. The peaks shown in Figure 3.9 (a) at the same retention times as the ions *m/z*=577, 865, 1153, and 1441 were numbered as peak 6, 7, 8 and 9, presumed to be the corresponding procyanidin oligomers.

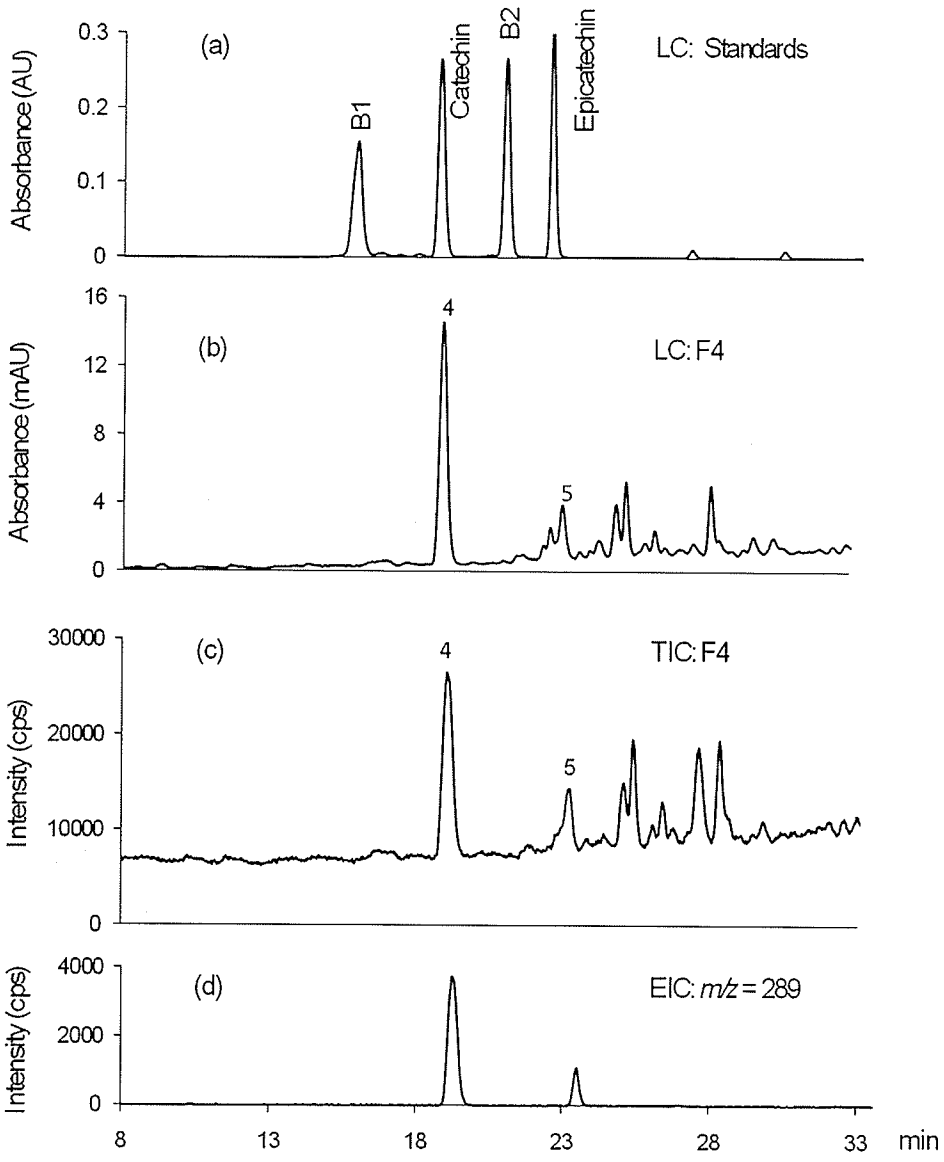


Figure 3.8. (a) LC chromatogram (at 280 nm) of standards; (b) LC chromatogram (at 280 nm) for F4 extracted from Manomin wild rice; (c) Total ion chromatogram (TIC) of F4; (d) Extracted ion chromatogram (EIC) of a single ion at *m/z* 289.

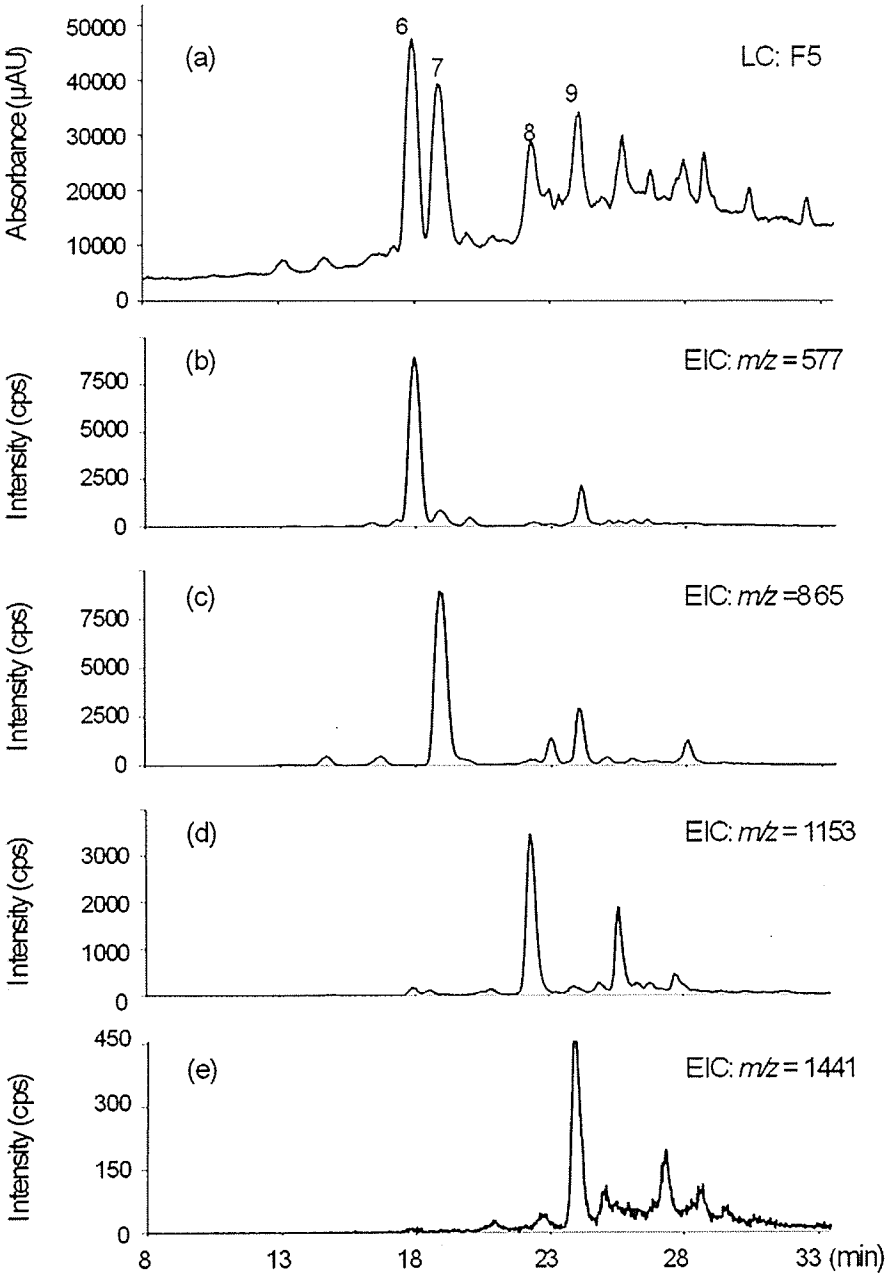


Figure 3.9. (a) LC chromatogram at 280 nm of F5 for Manomin wild rice; (b-e) Extracted ion chromatograms (EIC) of deprotonated molecules with (c) *m/z* = 577, (d) *m/z* = 865, (e) *m/z* = 1153, and (f) *m/z* = 1441.

The identification of these peaks was further completed by using tandem mass analysis. As shown in Table 3.4, the fragmentation characteristics of the numbered peaks were consistent with the previous reports on procyanidin oligomers (Cooper et al, 2007). Hence, the previous assumption has been confirmed. Peak 6, 7, 8 and 9 were identified as dimer, trimer, tetramer, and pentamer of procyanidins, respectively.

In contrast to wild rice, no procyanidins were detected in the white rice control. The absence of such constituents may explain the low antioxidant activity of the control.

Table 3.4. Identification of catechins and procyanidin oligomers in wild rice fractions (F4 and F5) by LC-MS/MS in negative ion mode

Peak no.	Compound assignation	Fraction no.	t_R (min)	$[M-H]^-$ *m/z*	Product ion *m/z*
4	(+)-Catechin	F4	19.22	289	245
5	(-)-Epicatechin	F4	23.50	289	245
6	Dimer	F5	17.88	577	407, 289
7	Trimer	F5	18.82	865	577, 407, 289
8	Tetramer	F5	22.26	1153	865, 407, 289
9	Pentamer	F5	23.96	1441	1153, 865, 577

3.3.7. Quantification of catechins and oligomeric procyanidins in F4 and F5

Catechin monomers (catechin and epicatechin) were commonly found in all the wild rice samples, whereas the presence of procyanidin oligomers varied among samples. To quantify these flavan-3-ols in wild rice, (+)-catechin was used as the standard for all the peaks due to the lack of oligomeric procyanidin standards. The contents of procyanidins in wild rice samples are shown in Table 3.5 and expressed as mg catechin equiv per kg. The term "trace" used in the table represents the compounds that were detected by mass analysis, but could not be quantified due to absence of signals in the UV chromatograms.

Among raw samples, Manomin wild rice contained the highest procyanidin content (239 mg/kg), followed by small sized wild rice (162 mg/kg) and B black wild rice (162 mg/kg). The lowest total content of procyanidins (7mg/kg) was found in hand harvested wild rice because only monomers were detected in this wild rice, with the presence of minor dimer. Procyanidin oligomers (DP>3) were also absent in C scarified and Canadian wild rice which resulted in their low procyanidin content (36 and 24 mg/kg, respectively). The other raw samples were found to contain oligomeric procyanidins up to pentamer among which trimers were the most abundant. Compared with raw samples, the processed sample, quick cooking wild rice contained a relatively low procyanidin content of 24 mg/kg due to the absence of trimer, tetramer and pentamer. The mixed sample was also found to be low in procyanidin content (36 mg/kg).

Table 3.5. Content of procyanidin monomers and oligomers in wild rice [a]

		Procyanidin oligomers (mg per kg)					
Group	Sample name	Mono- [b]	Di-	Tri-	Tetra-	Penta-	Total
Control	White rice	nd	nd	nd	nd	nd	Nd
Mixed	3 blend mix	6.64	11.99	17.31	nd	nd	35.93
Processed	Quick cooking	12.43	11.91	nd	nd	nd	24.34
Raw	A black	13.46	23.87	49.41	31.47	30.96	149.17
	B black	13.56	23.88	52.18	34.12	37.37	161.10
	C scarified	18.15	17.42	trace	nd	nd	35.57
	Canadian lake	11.42	12.42	trace	nd	nd	23.84
	Hand harvested	7.16	trace	nd	nd	nd	7.16
	Minnesota cultivated	12.46	25.26	55.03	29.71	32.23	154.70
	Manomin	13.74	42.42	69.18	52.67	61.62	239.22
	Large size	9.27	22.88	35.85	32.98	43.43	144.41
	Small size	11.17	24.47	34.45	34.08	57.74	161.91

[a] Data are the averages of duplicate tests.

[b] The monomer content comprises catechin and epicatechin.

3.3.8. Correlation between antioxidant activity and TPC in wild rice crude extracts

To assess the contribution of TPC to total antioxidant activity of wild rice, the relationship among TPC, DPPH free radical scavenging activity, and ORAC were investigated. A high correlation ($r=0.92$, $P<0.0001$) was found between TPC and DPPH radical scavenging activity. TPC and ORAC were also correlated ($r=0.64$, $P<0.05$). Therefore, the higher

antioxidant activity of wild rice with respect to white rice is attributed to the larger amount of total phenolics in the former.

3.4. Conclusions

The acidic acetone extracts of wild rice displayed up to 30 times higher antioxidant activity than that of white rice control by using DPPH photometric method and ORAC method, which signifies that more health benefits may be obtained by consuming wild rice than white rice. The processed sample, quick cooking wild rice and the mixed sample exhibited lower antioxidant activity compared with raw samples. The total phenolic contents in wild rice, about15 times greater than white rice, were demonstrated to correlate with the antioxidant activity of wild rice. The techniques of HPLC and LC-MS/MS allowed the detection of flavones and favan-3-ols in wild rice after fractionation of crude acetone extracts. The pronounced antioxidant activities of F2 and F3 were attributed to glycosylated flavones designated as di-hexosyl apigenin, hexosyl-pentosyl apigenin, and di-pentosyl apigenin. Catechin, epicatechin and oligomeric procyaninds were the predominant antioxidants detected in F4 and F5. The above flavonoid compounds absent in white rice may explain its low antioxidant activity.

GENERAL CONCLUSIONS

Wild rice contained exclusive phenolic compounds and exhibited significantly higher antioxidant activity than white rice ($p<0.05$). The DPPH radical scavenging activity and oxygen radical absorbance capacity (ORAC) of wild rice were respectively up to 5 and 6 times greater than that of white rice by using methanol extraction, while up to 15 times greater by using acetone extraction. A comparison of the antioxidant activites of methanol extracts and acetone extracts from wild rice showed that methanol extracts had higher ORAC values, whilst lower in DPPH radical scavenging activity. The total phenolic content (TPC) of methanol extracts and acetone extracts were comparable. Mixed and processed wild rice showed significantly lower antioxidant activities than raw samples, with reduced TPC levels.

The alkaline hydrolysates of methanol extracts and the remaining residues contained a range of phenolic acids, with ferulic acid exhibiting the highest concentration among the acids. Sinapic acid was the second most abundant phenolic acids in wild rice followed by *p*-coumaric acid. They were predominantly present in the insoluble bond form. The significant amounts of ferulic acid and sinapic acid gave rise to their dehydrodimers. The identified ferulate dehydrodimers included 8-8', 8-5', 8-O-4', and 5-5' coupled isomers, with 8-O-4' coupled dimers being the most abundant. Two 8-8' coupled sinapate dehydrodimers were also detected in wild rice with the noncyclic isomers as the predominant form.

The acetone extracts from wild rice were characterized by flavonoid compounds, which were predominantly present in glycosylated form. The major flavonoid glycosides identified in wild rice consisted of di-glucosyl apigenin, glucosyl-arabinosyl apigenin, and di-arabinosyl apigenin. Proanthocyanidins (flavan-3-ols) were also detected in wild rice including catechin,

epicatechin and oligomeric procyanidin. These phenolic compounds significantly contributed to the antioxidant activity of wild rice.

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