VARIETAL DIFFERENCES IN ANTIOXIDANT ACTIVITY AND PHENOLIC COMPOSITION OF ASPARAGUS

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of ESRA CAKIR find it satisfactory and recommend that it be accepted.

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VARIETAL DIFFERENCES IN ANTIOXIDANT ACTIVITY AND PHENOLIC COMPOSITION OF ASPARAGUS

Abstract

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The study was focused on two areas of inquiry. The first involved analyzing nine varieties of asparagus; 'Jersey Knight', 'Jersey Deluxe', 'Jersey Supreme', 'Jersey Giant', 'Guelph Millenium', 'UC 157', 'Purple Passion', 'Morehouse Select' and 'Syn4' for total phenolics (TPH), rutin content, and antioxidant activity using Folin-Ciocalteu procedure, colorimetric $AlCl_3$ method, and the 1,1 diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay, respectively. TPH of varieties ranged from 29.09 to 35.16 mg rutin equivalent/g dry weight basis (dwb) while rutin content varied from 12.09 to 16.09 mg/g dwb. TPH levels were well correlated with rutin content ($R \geq 0.90$, $p < 0.05$). Rutin contributed approximately 45% of total phenolics. 'Guelph Millenium' contained the greatest TPH and rutin contents while Purple Passion had the least. DPPH scavenging activity of asparagus varieties ranged from 10.70 to 15.30 mg rutin/g dwb. Secondly, asparagus extracts were analyzed by reversed-phase high performance liquid chromatography (HPLC) equipped with a diode array detector to determine the content and profile of phenolics. Rutin was identified as the major phenolic compound, followed by chlorogenic acid, in all varieties. HPLC coupled to a radical scavenging method was

applied in order to determine antiradical components in the asparagus extracts. The determination of antiradical activity was based on a decrease in the peaks correspondent to strong antioxidants, 60 min after the addition of DPPH. A green (‘Guelph Millenium’) and a purple (‘Purple Passion’) asparagus variety were analyzed to identify the variations in different colors of asparagus. Rutin was found to be the strongest radical scavenging component. After the addition of DPPH solution, the peaks corresponding to rutin and chlorogenic acid decreased by 93% and 72% of the initial peak intensity, respectively. Purple asparagus had a unique compound different than green asparagus varieties. This unknown compound did not possess any antiradical activity. Total anthocyanin contents of asparagus varieties were measured spectrophotometrically. ‘Purple Passion’ contained the largest total anthocyanin content (1.08 mg/g dwb) among the varieties tested. These results provide useful information about asparagus varieties having valuable potential health benefits.

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INTRODUCTION

Washington Sate produces about 35 percent of the asparagus grown in the United States on a total of 13,000 acres of harvest area, second only to California. The Washington industry is mainly concentrated on fresh market, producing about 30 million pounds of asparagus each year. The prime harvest season in Washington is from mid-April to mid-June. Both acreage and production rate have declined in recent years due to high labor costs and competition from foreign markets. The overall objective of the "Improving the International Competitiveness of the Washington/Oregon and Michigan Asparagus Industries" project is to find prospective solutions to the problems of the asparagus industry. The main foci of the project are: reducing the costs of production through alternative production and harvesting (mechanical) methods, developing new and more efficient methods of handling asparagus from harvest through fresh packing or processing, designing innovative processes to enhance the shelf-life and quality of fresh asparagus, exploring alternative processing techniques that add value and reduce waste of by products and identify nutritional/antioxidant benefits unique to locally produced asparagus.

Asparagus is known as a rich source of phytochemicals. Previous research suggested that asparagus ranked 1st in total phenol antioxidant index (based on dry weight) among 23 popular vegetables (Vinson et al., 1998). Recently Pellegrini *et al*. (2003) showed that asparagus had the highest antioxidant activity among 34 vegetables commonly consumed in Italy. Prior and Wu (2007) reported that asparagus has a strong hydrophilic antioxidant capacity compared to other vegetables. However, a number of

factors, including variety, growing conditions, location and season may affect the levels of phenolic compounds and thus, antioxidant activity.

The objective of this study was to evaluate the influence of variety and harvest date on polyphenolic composition and associated antioxidant activities of asparagus grown in Washington State. The study investigated the differences in the total phenolic, and rutin contents as well as antioxidant capacities of asparagus varieties by spectrophotometric assays. Asparagus varieties were also analyzed by reversed-phase high performance liquid chromatography (HPLC) equipped with a diode array detector after a multi-step extraction process to determine the content and profile of phenolics. The anthocyanin content of the extracts was measured spectrophotometrically. In addition, individual antiradical compounds and their efficiency were determined using HPLC coupled to a radical scavenging method.

LITERATURE REVIEW

A. ASPARAGUS

1. Classification and History

Asparagus (*Asparagus officinalis L.*), a perennial plant, is a member of the family Liliaceae. Asparagus is believed to have originated in the eastern Mediterranean and eastward to the Caucasus Mountains. It was consumed as both food and medicine by the ancient Greeks, but the cultivation of asparagus did not start until the Roman Empire. In the time of Cato (about BC 200), the cultivation methods of asparagus were developed (Peirce, 1987). Asparagus became popular in Europe during the sixteenth century. During this time, some of the varieties started to be selected for cultivation. 'Violet Dutch' was the first variety that gained international importance. The majority of asparagus cultivars developed by breeding studies originated from 'Violet Dutch' (Knaflewski, 1996). Asparagus was brought to America by early colonists in the 1600's. However, asparagus was not produced commercially in the United States until the mid-19th century (Decoteau, 2000).

2. Plant Characteristics

Asparagus is a monocotyledonous, herbaceous perennial, 4 to 6 feet tall. The underground part of the plant, the crown, consists of rhizomes, fleshy roots and fibrous roots. Fleshy roots are known as carbohydrate storage roots that support spear growth in the spring. Fibrous roots are responsible for the absorption of nutrients and water. Fleshy roots die after several years of function and regenerate from the crown, while fibrous roots develop each year from the fleshy storage roots (Rubatzky and Yamaguchi, 1997; Peirce, 1987). The rhizome provides the transfer of carbohydrates from fleshy roots to the above

ground portion. Edible spears arise from the elongated buds on the rhizome. Bud size directly affects the spear size; the larger the buds, the larger the spears. The cladophyll, the modified stem, is the photosynthetically active part of the spear. Along the spear, there are also some lateral branches extending from nodes under the bud scales. Although these fernlike branches seem like leaves, they are actually modified stems. The triangular shaped bud scales are the true leaves that have no photosynthetic function (Rubatzky and Yamaguchi, 1997). As spears grow, lignin starts to accumulate at the base of the spear. If the spears are not harvested, they then continue with vigorous fern growth (Cantaluppi and Precheur, 1993).

The color of asparagus spears can be white, green, or purple. The purple color is due to the anthocyanin pigment. The green and white asparagus can be the same variety, but the growing methods are different. Green asparagus spears are exposed to direct sunlight as soon as they emerge from the soil, while white asparagus is grown in the dark (Decoteau, 2000). When asparagus is exposed to ultraviolet light, spears first turn to pink, then acquire their familiar green color. However, in white asparagus production, black “polyhouses” are built over the plant to keep spears away from the sunlight. At the end of the season, the "polyhouses" are removed to allow the crop to continue its growth and produce the asparagus fern (Cantaluppi and Precheur, 1993).

Asparagus is a dioecious plant, having male or female reproductive structures on different plants. The number of occurrence of each sex in traditional cultivars is approximately equal. The male blossoms are slender, yellowish green and more conspicuous than female blossoms (Thompson, 1931). Although female plants produce larger spears than males, the energy consumed for seed production reduces the yield of

spears. Many new all-male hybrid asparagus varieties have been developed to enable the plant to spend its energy only on spear production, instead of growing seed, thus increasing yield (Decoteau, 2000).

3. Climatic requirements

Asparagus is a cool season crop producing the maximum number of spears when the mean day temperature is between 25-30°C. Asparagus needs a long rest period and an average summer growing temperature of 66-75°F for successful spear production. Spear production is slow when the average daily temperature is 50°F or below. However, spears tend to branch quickly upon emergence when the temperature is 100°F or higher. When temperatures are between 75-80°F, a spear approximately six inches in length is produced in two days (Peirce, 1987; Rubatzky and Yamaguchi, 1997).

4. Varieties of asparagus

In the beginning of the 20th century, J.B. Norton developed Washington varieties by crossing a male plant of an unknown American asparagus with a female plant of 'Reading Giant' from England. As a result of this study open pollinated cultivars, 'Mary Washington', 'Martha Washington' and 'Waltham Washington' were developed. The purpose of this breeding study was to develop cultivars resistant to rust (*Puccinia asparagi*), which had threatened the asparagus industry in North America at the end of the nineteenth century. 'Mary Washington' gained more popularity for its larger spears, tighter heads and better rust tolerance (Thomson, 1931; Knaflewski, 1996). However, in dioecious cultivars, flowers of two sexes have to cross-pollinate which causes significant genetic variation in vigor and appearance (Peirce, 1987). A pure stock of open-pollinated

Washington varieties was not retained so they lost most of their characteristics and they are no longer recommended for commercial production (Knaflewski, 1996). Many other strains grown worldwide originated from 'Mary Washington' including well-known cultivars developed in California and New Jersey.

Several open-pollinated cultivars such as 'UC309', 'UC500', 'UC711', 'UC873', 'UC500W', 'UC66' and 'UC72' were bred at UC Davis for warm and arid climates. The spear tips of these Californian hybrids remain tightly closed until the spear is tall (8-9"), resulting in high level of spear quality under warm harvest temperatures (Benson et al., 1996). A clonal hybrid 'UC 157', one of the most popular varieties for warm climates, was selected from 'Mary Washington' and 'UC500' by the breeding program at the University of California Riverside-Davis (Roose and Stone, 1999). This variety gives good yield in warm and dry regions like California, Oklahoma, Mexico and Peru. However, due to the winter damage to the crown, its yield decreases after four or five years in colder regions (Cantaluppi and Precheur, 1993).

In 1984, new hybrids, 'Apollo', 'Atlas', and 'Grande' were developed by California Asparagus Seed & Transplants (C.A.S.T), Inc. The objective was to have varieties that produce taller spears without the tips opening up under high temperatures. When the spear tip opens up or "ferns out", lignin builds up at the base of the spear, causing it to become tough or woody. The female parent of those varieties was selected from 'UC 157', which has high quality at warm climates. The male parent was selected from a New Jersey supermale, for its high yield and adaptability to colder regions. Yield trials showed that these hybrids outyielded 'UC 157' by about 25% in California and gave better results in northern growing areas such as Michigan and Washington (Benson et al., 1996).

‘Purple Passion’ is another C.A.S.T. hybrid, selected from ‘Violetto d'Albinga’ from the western Mediterranean coast of Italy. This new tetraploid variety has an attractive purple color with creamy white interior. It produces very large spears containing about 20% more sugar than green asparagus (Benson et al., 1996). It has a mild and nutty flavor and is generally preferred raw in salads. Its’ striking purple color turns to green when it is cooked. Although this variety produces very vigorous plants, it is more susceptible to asparagus rust (Facciola, 1990)

Dr. Howard Ellison and Dr. Stephen Garrison at Rutgers University, New Jersey, have focused their research on breeding all-male hybrids. In dioecious cultivars male and female flowers are found on different plants. Male flowers always possess a dominant male (M) gene. This gene can be found in either heterozygous (Mm) or homozygous (MM) form. However, the occurrence of homozygous males is very rare in nature (Cantaluppi and Precheur, 1993). Dr. Ellison was able to produce several homozygous (MM) males, which is also known as supermale, by selfing hermaphrodites (male plants with functional female flower parts). The progeny of a heterozygous male is half male and half female, while the progeny of a supermale contains only males. All-male hybrid (Mm) is produced when a tissue-cultured supermale (MM) is crossed with a female (mm) (Garrison and Chin, 2005).

These new all-male hybrids have brought many advantages to asparagus growers. All-male hybrids provide superior vigor, higher yields, better rust resistance and tolerance to Fusarium crown rot than female plants. The main benefit of an all-male hybrid is that it doesn't produce berries or seed, which can later cause weed problems. Since there is no seed production, all of the nutrients for the seed growth is used by the crown and roots to produce larger plants (Knaflewski, 1996).

The first all-male hybrid released by Rutgers University was ‘Jersey Giant’. Soon after being released, ‘Jersey Giant’ gained worldwide popularity because of its high productivity and wide adaptation. Yield trials from Washington State to New England and South Carolina showed that this variety has consistently greater yields over the years than standard (dioecious) varieties. ‘Jersey Giant’ is resistant to rust and tolerant to Fusarium root and crown rot. It produces large and very attractive green spears with purple bracts and tight tips (Ellison and Kinelski, 1985).

Twenty additional all-male hybrids with excellent resistance to Fusarium were patented by the Rutgers Asparagus Breeding program, including ‘Jersey Knight’, ‘Jersey King’, ‘Jersey Supreme’, ‘Jersey Gem’, ‘Jersey Deluxe’, ‘Jersey Prince’, ‘Jersey Jewel’, ‘Jersey General’, and ‘Jersey Titan’. ‘Jersey Knight’ is one of the most popular all-male hybrids, having many of the same fine characteristics as the ‘Jersey Giant’ (Garrison and Chin, 2005). It has adapted to temperate, warm and cool climates and performs well in heavy clay soils. This all-male hybrid provides very high tolerance to fusarium and rust and is considered to be a good variety for replanting asparagus beds because of disease resistance. Spear size and color is comparable to the ‘Jersey Giant’, produces a large, thick, flavorful green spear with purple bracts of excellent quality (Facciola, 1990).

The breeding program at the University of Guelph, Canada also gave rise to the development of new varieties adapted to cold growing conditions. The ‘Viking’ series were one of the open-pollinated strains bred from ‘Mary Washington’ in Ontario, Canada. Although they yield higher than ‘Mary Washington’, most of the open-pollinated varieties yield less than the all-male New Jersey hybrids. A newer all-male hybrid was produced at University of Guelph called ‘Guelph Millenium’. It is a superb variety, producing high

yields of top quality green spears and is well adapted to damper climates, where Jersey hybrids have had winter kill at temperatures of -30ºF with no snow cover (Munro and Small, 1997).

A synthetic form of 'Jersey Giant' was also developed from one seed parent and two pollen parents. This synthetic hybrid, called 'Syn 4', is comparable to 'Jersey' hybrids in most aspects like productivity, vigor, high quality and resistance to rust but it is a mixture of 70% male and 30% female (Facciola, 1990).

5. Production and Marketing

The United States is the world's third leading producer of asparagus after China and Peru, with 138 million pounds for fresh market and 71 million pounds for processing. The U.S. produces mainly green asparagus and the commercial production occurs primarily in California, Washington and Michigan. According to the USDA's National Agricultural Statistical Service (NASS), California is the leading state with 46% of the 52,500 total U.S. acres of asparagus harvested in 2004. Washington and Michigan each produced approximately 27% of harvested acreage. However, Washington has provided the highest yields per acre followed by California and Michigan (USDA, 2004). The Washington industry is primarily focused on fresh market, producing about 30 million pounds fresh asparagus each year. The prime harvest season in Washington is from mid-April to mid-June. Washington asparagus is produced mostly in Franklin county (40.9%) and the Yakima Valley (32.8%) on a total of 13,000 acres of harvest area (Ball et al., 2002; USDA, 2005).

U.S. asparagus production and market share have been declining due to the intense competition from Peru and Mexico. The Peruvian asparagus industry is taking advantage of favorable labor costs and high yields of produce in seasons when neither U.S. nor Mexican producers can harvest. The Peruvian producers have also benefited from reduced tariffs provided by the Andean Trade Promotion and Drug Eradication Act (ATPDEA) of 2002, which was aimed at reducing Peruvian coca production as a major part of U.S. anti-drug efforts (Boriss, 2006).

B. PHENOLIC COMPOUNDS

1. Plant phenolics

Phenolic compounds or polyphenols are secondary metabolites found widely spread throughout the plant kingdom. Phenolics are a class of chemical compounds consisting of a hydroxyl group (-OH) attached to an aromatic hydrocarbon group. Phenolic compounds are generally derived from phenylalanine via the shikimate pathway (Ribereau-Gayon, 1972). Polyphenols can be found as simple phenolic molecules, such as phenolic acids or polymerized into larger molecules such as the proanthocyanidins (condensed tannins) and lignins (Bravo, 1998). Natural polyphenols are primarily found conjugated with one or more sugar residues linked to hydroxyl groups. Phenolic compounds can be classified into several subclasses as shown in **Table 1**.

Table 1. Classes of phenolic compounds in plants (Harborne, 1999)

Class	Structure
Simple phenolics, benzoquinones	C_6
Hydroxybenzoic acids	$C_6 - C_1$
Acethophenones, phenylacetic acids	$C_6 - C_2$
Hydroxycinnamic acids, phenylpropanoids (coumarins, isocoumarins, chromones, chromenes)	$C_6 - C_3$
Napthoquinones	$C_6 - C_4$
Xanthones	$C_6 - C_1 - C_6$
Stilbenes, anthraquinones	$C_6 - C_2 - C_6$
Flavonoids, isoflavonoids	$C_6 - C_3 - C_6$
Lignans, neolignans	$(C_6 - C_3)_2$
Biflavonoids	$(C_6 - C_3 - C_6)_2$
Lignins	$(C_6 - C_3)_n$
Condensed tannins (proanthocyanidins or flavolans)	$(C_6 - C_3 - C_6)_n$

Flavonoids are the most widely distributed plant phenolics, with more than 4,000 flavonoids identified in fruits, vegetables, nuts, seeds, flowers and roots (Merken and Beecher, 2000). Flavonoids are low molecular weight compounds, consisting of a diphenylpropane skeleton (C_6-C_3-C_6). The structure, as shown in **Figure 1**, is composed of two aromatic rings, A and B linked through three-carbon bridge, in the form of oxygenated heterocycle, C. The differences in the oxidation level or substitution pattern of the C ring subdivide the flavonoids into major subclasses: flavones (basic structure), flavonols (having a hydroxyl group at the 3-position), isoflavones (B ring binds to the 3-position), flavanones (2-3 bond is saturated), and catechins (C-ring is 1-pyran), chalcones (C-ring is opened), and anthocyanidins (C-ring is 1-pyran, and 1-2 and 3-4 bonds are unsaturated) (Balasundram et al., 2006). Flavonoids are most commonly found in plants as *O-glycosides* with sugars attached at the C3 position (Hertog et al., 1992). The sugars generally occur in the form of hexoses such as glucose, galactose, and rhamnose or pentoses such as arabinose and xylose. The sugars can be attached individually or in combination with each other. Glycosylation increases the water solubility of the molecule and allows its accumulation in the vacuoles of cells (Rice-Evans et al., 1997). Quercitrin, rutin, and robinin are the most common flavonoid glycosides in the diet. They are hydrolyzed by intestinal flora to produce the biologically active aglycone (Kijhnau,1976).

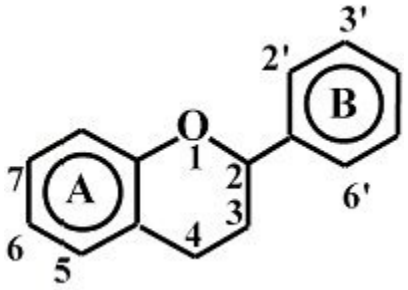

Figure 1. Basic structure of a flavonoid molecule

Among the flavonoids, flavones (e.g. apigenin, luteolin, diosmetin), flavonols (e.g. quercetin, myricetin, kaempferol) and their glycosides are the most common compounds. Flavonols and flavones differ from each other by the presence of a hydroxyl group at C3 in flavonols. Quercetin glycosides are the most widely distributed flavonoid in vegetables and fruits, while glycosides of kaempferol, myricetin, luteolin and apigenin exist in trace amounts (Hertog et al., 1992). Flavanols (catechin) are present mainly in tea. The concentrations of catechins are higher in green tea, while the oxidation undergone by the leaves in black tea processing converts these simple flavonoids into theaflavins and thearubigins (Cabrera et al., 2006). Flavanones constitute the majority of flavonoids in citrus fruits, where they present as mono- and diglycosides. All orange-type citrus fruits contain the flavanone aglycones hesperetin and naringenin, but they rarely occur as aglycones in the fruit itself. The dominant flavanone glycosides in sweet oranges are hesperidin and narirutin, whereas in sour oranges neohesperidin and naringin predominate (Peterson et al., 2006). Isoflavones (e.g., genistein, daidzein) are especially found in legumes. Anthocyanins are the most important group of water soluble plant pigments and are responsible for the orange, red, blue and purple color of many fruits and vegetables, such as apples, berries, beets and onions (Shi et al., 2005). They are glycosides or acylglycosides of anthocyanidins. Although seventeen anthocyanidins are found in nature, only 6 of them, cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), pelargonidin (Pg), and malvidin (Mv), are common in fruits. Anthocyanins are used as natural food colorants, because of their intense coloring ability (Wu et al., 2006).

Simple phenols and flavonoids constitute the biggest portion of the phenolic compounds. Their solubility depends on the differences in their polarity and chemical structure such as degree of hydroxylation, glycosylation or acylation.

2. Antioxidant activities of plant phenolics

Autooxidation is a free radical chain reaction and can be described in terms of initiation, propagation and termination processes. In the body, the oxidation of free radicals may contribute to a number of chronic and degenerative diseases such as cancer cardiovascular diseases, atherosclerosis, diabetes, and cataracts as well as the process of ageing (Shahidi and Naczk, 2003). Epidemiologic studies suggest that consumption of flavonoid-rich foods, in particular fruits and vegetables, is associated with a lower incidence of hearth diseases, ischemic stroke, cancer and other chronic diseases (Arts and Hollman, 2005). A study on 805 elderly Dutch males, aged 65 to 84 years, showed that coronary heart disease is inversely correlated with flavonoid intake in the diet (Hertog et al., 1993). Additional studies indicate a negative association between fruit and vegetable intake and the risk of colorectal cancer (Flood et al., 2002). Since those chronic diseases are associated with increased oxidative stress, it has been suggested that the protective effects of polyphenolic components is related to their antioxidative properties (Lotito and Frei, 2006). Cao et al (1998) observed an increase in the human plasma antioxidant capacity after consumption of diets rich in fruits and vegetables.

The antioxidant activities of phenolic compounds mainly depend on their free radical scavenging abilities, which is determined by their reducing properties as hydrogen-or electron-donating agents. Flavonoids are very effective scavengers of

hydroxyl and peroxyl radicals, although their efficiency as scavengers of the superoxide is not clear yet (Bravo, 1998). In addition, they have a metal chelation potential and inhibit the Fenton and Haber-Weiss reactions which produce active oxygen radicals (Rice-Evans et al., 1997). An important characteristic of flavonoids is that they can maintain their free radical scavenging capacity after forming complexes with metal ions. The following scheme illustrates the interference of an oxidation reaction by donation of a hydrogen atom from phenolic compound to radicals (Bravo, 1998):

$$ROO\cdot + PPH \rightarrow ROOH + PP\cdot$$

$$RO\cdot + PPH \rightarrow ROH + PP\cdot$$

There are two primary conditions to be able to define a polyphenol as an antioxidant: first, it should delay or prevent the oxidation of the substrate when they are present in low concentration compared to the oxidizable substrate; second, it should form stable phenoxy radical intermediates that act as terminators of the propagation step by reacting with other free radicals (Rice-Evans et al., 1996).

$$ROO\cdot + PP\cdot \rightarrow ROOPP$$

$$RO\cdot + PP\cdot \rightarrow ROPP$$

Many *in vitro* studies have pointed out the strong antioxidant activity of polyphenols due to their low redox potential and their capacity to donate several electrons or hydrogen atoms. However, the role of polyphenols *in vivo* is not clear. The antioxidant potential of the polyphenols depends on the extent of absorption and metabolism of these compounds. *In vivo* studies show that flavonoids are poorly absorbed and only very low levels were found in human plasma after the ingestion of large amounts of flavonoid-rich foods (Lotito and Frei, 2006). On the other hand, human studies and epidemiological data

suggest that these concentrations may be sufficient to yield a potent antioxidant action (Bravo, 1998).

3. Structure–activity relationship

The chemical structure of phenolic compounds is a key factor that affects their radical scavenging and metal chelating activity. This phenomenon is known as structure-activity relationship (SAR) (Seyoum et al., 2006). Phenol itself does not possess an antioxidant capacity, while *ortho*- and *para*- diphenolics exert activity, which enhances with hydrogen atom substitution (Balasundram et al., 2006). The main three structural elements involved in the antiradical activity are:

1. the degree of hydroxylation and positions of the hydroxyl groups (the o-dihydroxy group) in the B ring which confers higher stability to the radical form;
2. the double bond between C-2 and C-3 in conjugation with the 4-oxo group;
3. the hydroxyl groups at the 3'- and 5'- positions of the B ring (Rice-Evans et al., 1996).

The antioxidant activity efficiency of flavonoids is correlated with their degree of hydroxylation and decreases with the presence of a sugar moiety. Glycosylation at important hydroxyl positions affects the antioxidant activity of the flavonoids. Glycosides of phenolic substances are weaker antioxidants than the corresponding aglycones (Rice-Evans et al., 1997).

4. Methods to determine antioxidant activity

A number of spectrophotometric methods are currently used for determination of antioxidant activity of plant extracts. These assays differ from each other in terms of substrates, reagents, experimental conditions, reaction medium, standards, and analytical evaluation methods. The exact comparison of the results and their general interpretation are practically impossible due to the variability of experimental conditions and differences in physicochemical properties of oxidizable substrates.

The applied antioxidant capacity methods can be divided into two basic categories due to the chemical reactions involved: (i) hydrogen atom transfer (HAT) reaction based assays and (ii) single electron transfer (ET) reaction based assays. The first category uses competitive reaction kinetics and the quantification is derived from kinetic curves, while the second category includes a redox reaction with the oxidant as an indicator of the reaction endpoint. Both types of assays were developed to measure the radical scavenging capacity instead of the preventative antioxidant capacity of a sample (Huang et al., 2005).

4.1. HAT - Based Assays

These assays are based on the hydrogen atom donating capacity of antioxidants. In general, HAT-based assays are composed of a synthetic free radical generator, an oxidizable molecular probe and an antioxidant. Added antioxidant competes with probes for the radicals and thus inhibits or retards the oxidation of the probes (Shahidi and Zhong, 2007).

a. Oxygen Radical Absorbance Capacity Assay (ORAC)

The ORAC assay uses a peroxyl radical induced oxidation reaction to measure the antioxidant's chain breaking ability. The fluorescence decay of the probe indicates its oxidation by peroxyl radical, which is detected by a fluorometer. Initially this method was developed by Cao et al (1993) using B-phycoerythrin (B-PE) as the probe. B-PE was later replaced with fluorescein (FL:3'6'-dihydroxyspiroisobenzofuran-1[3H],9'[9H]-xanthen]-3-one) because of several disadvantages of B-PE such as its large lot-to-lot variability, its reaction with polyphenols due to nonspecific protein binding and its being photobleached under excitation light (MacDonald-Wicks et al., 2006). In general, sample, control and standards (a series of Trolox solutions) are mixed with the FL solution and incubated at 37 ^{o}C before AAPH (2-2'-azobis (2-amidinopropane) dihydrochloride: peroxyl radical generator) initiates the reaction. As the FL is consumed by the reaction the fluorescence intensity decreases. Antioxidants scavenge peroxyl radicals and inhibit the loss of fluorescence intensity, which defines their activity. The ORAC values are obtained by a net integrated area under the fluorescence decay curves (AUC = AUC_{sample} – AUC_{blank}) and a standard curve (Trolox concentration versus AUC) (Shahidi and Zhong, 2007).

b. Total Radical-trapping Antioxidant Parameters (TRAP)

The TRAP assay was designed to measure *in vivo* antioxidant activity of human plasma by the reaction between peroxyl radicals and a target probe. Peroxyl radicals are produced from an azo initiator, ABAP (2,2'-diazobis-(2-amidinopropane) dihydrochloride), by thermal decomposition. Several reaction probes have been applied

to monitor the reaction such as oxygen consumption, fluorescence of R-phycoerythrin (R-PE) and absorbance of ABTS (2,2'-azobis-(3-ethylbenzothiazoline-6-sulfonic acid). Antioxidant activity is expressed as Trolox equivalent by comparing the lag time of the kinetic curve in the presence of the antioxidant with the presence of Trolox standard (Huang et al., 2005)

Although TRAP is a useful assay for antioxidant activity measurement, the precision and reliability of the method is problematic due to the fact that antioxidant's activity can continue after the lag phase (Shahidi and Zhong, 2007).

4.2. ET - Based Assays

These assays are based on the following electron transfer reaction;

Probe (oxidant) + e (from antioxidant) → *reduced probe + oxidized antioxidant*

The degree of color change of the probe by the oxidation is proportional to the antioxidant concentrations. Since there is no oxygen radical in the equation, it is questionable to relate the results to antioxidant activity *in vivo* systems. These methods are basically based on the assumption that antioxidant activity is equal to its reducing capacity. ET-based assays are commonly used because of the speed and ease of the assay (Huang et al., 2005; Shahidi and Zhong, 2007).

a. Trolox Equivalent Antioxidant Capacity (TEAC)

TEAC is a decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants. The oxidant, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)

($ABTS^{\bullet-}$) is generated by oxidation of $ABTS^{2-}$ with potassium persulfate and the radical is reduced to colorless $ABTS^{2-}$ in the presence of antioxidants (Re et al., 1999).

$$ABTS^{\bullet-} \xrightarrow[K_2SO_5]{\text{antioxidants}} ABTS^{2-}\ \text{(colorless)}$$

The degree of decolorization as percentage inhibition of $ABTS^{\bullet-}$ is determined as a function of the concentration of antioxidant and duration of reaction and compared with Trolox standards under the same conditions (Cano et al., 1998).

Although TEAC assay is classified as an ET-based method, there is a little correlation between the TEAC number and the number of electrons that an antioxidant can give away (Huang et al., 2005). Another problem is that $ABTS^{\bullet-}$ does not resemble the radicals found in biological systems. On the other hand, TEAC is widely used for antioxidant capacity assays because of its simplicity and automation.

b. 2,2 diphenyl-1-picrylhydrazyl (DPPH) radical assay

This assay uses a stable chromogen radical, $DPPH^{\bullet}$ in methanol solution, which gives a deep purple color. By the addition of antioxidants to the DPPH solution, the color of the solution fades and the reduction is monitored by the decrease in absorbance at 515nm (Brand-Williams et al., 1995). The antioxidant efficiency is calculated by the concentration of antioxidant required to decrease the initial DPPH concentration by 50% (EC_{50}) and the time necessary to reach the steady state with EC_{50} (Molyneux, 2004).

DPPH assay is a simple and widely used method. However, it has some weaknesses; unlike reactive peroxyl radicals involved in lipid peroxidation, DPPH is a long-lived nitrogen radical. In addition, antioxidants reacting quickly with peroxyl

radicals may react slowly with the DPPH. The reaction kinetics between the DPPH and antioxidants are not linear to the DPPH concentrations thus makes EC_{50} measurement problematic (Huang et al., 2005).

c. Ferric Ion Reducing Antioxidant Power (FRAP)

The FRAP assay measures the reduction of a ferric salt, $Fe(III)(TPTZ)_2Cl_3$ (TPTZ=tripyridyl-triazine), to the blue colored ferrous complex by antioxidants under acidic (pH 3.6) conditions. The increase in absorbance (ΔA) at 593 nm is measured and compared with ΔA of a Fe(II) standard solution. ΔA is linearly proportional to the concentration of antioxidant. One FRAP unit is defined as the reduction of 1 mol of Fe(III) to Fe(II) (Pulido et al., 2000).

The FRAP assay is a simple, economic and reproducible method which can be applied to both plasma and food extracts. This method has the advantage of determining the antioxidant activity directly in whole plasma, it is not dependent on enzymatic or nonenzymatic methods to generate free radicals prior to the evaluation of the anti-radical efficiency of plasma and no isolation of plasma fractions such as LDL is required (Pulido et al., 2000). However, potential problems occur, as the oxidant contains other Fe(III) species, which many metal chelators in food extract could bind to and form complexes that also react with antioxidants (Frankel and Meyer, 2000; MacDonald-Wicks et al., 2006).

d. Total Phenols Assay by Folin-Ciocalteu Reagent (FCR)

FCR, developed by Singleton and co-workers (1965), is the most widely applied procedure for quantification of total phenolics in plant extracts and beverages. Several studies point to a significant linear relationship between ET-based antioxidant activity assays and total phenols assay by FCR (Velioglu, 1998; Kahkonen, 1999). When the chemistry of the assays is compared, the similarity is not very surprising. FCR assay is based on the reduction of yellow heteropoly-phosphotungstates-molybdate anions to a blue colored complex in an alkaline solution, in the presence of phenolic compounds (Shahidi and Nackz, 2003). Thus, FCR assay basically uses an electron transfer reaction, where dissociated phenolic proton reduces the FCR (McDonald-Wicks et al., 2006).

The FCR is not very specific and it can be reduced by all phenolic compounds that exist in the extract such as extractable proteins. However it is simple, reproducible and convenient and it has become a routine assay in studying phenolic antioxidants (Huang et al., 2005).

4.3. Chromatographic methods

The phenolic content in plant materials can be measured by several separation techniques such as high-performance liquid chromatography (HPLC), gas chromatography (GC), capillary zone electrophoresis (CZE), etc. The chromatographic determination of phenolics is highly precise and accurate with a high informative value, but on some occasions it may be problematic to identify all of the phenolic compounds in a specific plant material. Chromatographic methods require small amounts of sample and

interference from minor compounds other than the selected product can be easily excluded (Strail et al., 2006).

Merken and Beecher (2000) reviewed the measurement of food flavonoids by HPLC. The most often used column in phenolic compound analysis have been packed with reversed phase C_{18} column material. The elution systems employed are usually binary, with an aqueous acidified polar solvent such as acetic acid, phosphoric acid or formic acid (solvent A) and a less polar organic solvent such as methanol or acetonitrile (solvent B). Detection of flavonoids is usually done by UV-vis with diode array detection (DAD). Recently, HPLC-MS and capillary electrophoresis methods have also been employed to determine phenolic compounds in foods. Mass spectrometry detectors coupled to HPLC (HPLC-MS tandem) provide structural characterization of phenolics (Shahidi and Naczk, 2003).

The effectiveness of antioxidants in complex heterogeneous foods and biological systems is affected by many factors. A number of different substrates, system compositions and analytical methods are used in testing protocols to evaluate the antioxidant capacity. As a result, the data obtained by different researchers are very difficult to compare and interpret. Evaluation of antioxidant activity therefore should be carried out under different experimental conditions of the oxidation reaction, using several methods to measure different products of oxidation related to real food quality or critical biological reactions and finally, the general trends of values for individual samples should be compared (Frankel and Meyer, 2000).

C. ANTIOXIDANT ACTIVITIES OF ASPARAGUS

Asparagus is recognized as a good source of phytochemicals for the human diet. Previous research has indicated that asparagus contains high levels of naturally occurring compounds such as flavonoids (Makris and Rossiter, 2001), carotenoids (Deli et al. 2000), thiols (Demirkol, et al. 2004), saponins (Pant et al, 1988), oligosaccharides and sulfur-containing compounds (Chin et al. 2002). The rich content of asparagus makes it distinctive among other common vegetables. The antioxidant activity of asparagus was one of the highest among 43 vegetables measured by a β-carotene bleaching method (Tsushida, et al., 1994). Asparagus was also ranked 1st in total phenol antioxidant index (based on dry weight) among 23 popular vegetables commonly consumed in the United States (Vinson et al, 1998). A more recent study suggested that asparagus had the greatest antioxidant capacity in the TRAP assay among 34 vegetables commonly consumed in Italy (Pellegrini et al., 2003). Asparagus is also rich in biological thiols which are known as important antioxidants. Glutathione and N-acetylcysteine contents of asparagus were the highest among several vegetables such as pepper, cucumber, spinach and broccoli (Demirkol et al. 2004). Glutathione protects cells against oxidative stress, regulates other antioxidants such as vitamin A and E and provides protection against certain forms of cancer in addition to potent anti-viral properties (Liu and Eady, 2005).

More than one hundred different kinds of foods, including fruits, vegetables, nuts, spices and cereals were analyzed for both lipophilic (L-ORAC$_{FL}$) and hydrophilic (H-ORAC$_{FL}$) antioxidant capacities using the ORAC assay (Wu, 2004). More than 96% of the total antioxidant capacity of asparagus was from H-ORAC$_{FL}$. However, the L-ORAC$_{FL}$ value of four specific vegetables including spinach, broccoli, asparagus and

lettuce was higher than that of other vegetables. After the cooking process, H-ORAC$_{FL}$ of asparagus decreased 44% compared to the raw forms.

The main phenolic in asparagus determined by LC/MS was rutin (Tsushida et al., 1994). Wang et al. (2003) reported that rutin content in asparagus varies among the spear and the youngest tissue, the tip, is the richest source for rutin at a concentration of 0.03-0.06% fresh weight basis. Antioxidant activity of asparagus reduces in food processing operations. Nindo et al. (2003) investigated the effect of several drying methods on nutritional value of asparagus. They reported that the tip portion of asparagus retained more antioxidant activity after drying than middle and basal parts. Sun et al (2005) reported that commercial pectolytic enzymes can change the phenolic composition and antioxidant activity of asparagus juice. It is reported that, in addition to rutin, asparagus contains simple phenols such as chlorogenic acid, caffeic acid and cinnamic acid (Sakakibara, 2005).

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CHAPTER ONE

ANTIOXIDANT ACTIVITY AND PHENOLIC CONTENT OF NINE VARIETIES OF ASPARAGUS

ABSTRACT

Asparagus is recognized as a good source of phytochemicals for the human diet. However, there may be varietal differences in composition and health benefits. Nine varieties of asparagus at three different harvest dates were evaluated for their total phenolic content (TPH), rutin content, and total antioxidant activity. Aliquots of aqueous extracts were analyzed for TPH using the Folin-Ciocalteu procedure. Rutin contents were determined colorimetrically using $AlCl_3$, and antioxidant activity was measured by the 1,1 diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay. TPH of varieties ranged from 29.09 to 35.16 mg rutin equivalent/g dry weight basis (dwb) while rutin content varied from 12.09 to 16.09 mg/g dwb. A significant correlation was found between TPH and rutin content ($R=0.90$, $p<0.05$). Results indicated that approximately 45% of TPH is rutin. 'Guelph Millenium' contained the greatest TPH and rutin contents while 'Purple Passion' had the least. The total antioxidant capacity of asparagus varieties ranged from a low of 10.70 to 15.30 mg rutin/g dwb measured by DPPH scavenging activity. These results provide useful information about asparagus varieties having valuable potential health benefits.

KEYWORDS: asparagus, antioxidant activity, DPPH, total phenolics, rutin

INTRODUCTION

Consumers are concerned not only with nutrient rich diets but also with minor food constituents that may benefit health. One category of these minor constituents is antioxidant phytochemicals. Current epidemiological studies have shown the consumption of vegetables and fruits have a protective effect on incidence of cardiovascular diseases (Bazzano et al., 2002) and certain cancers (Hertog et al., 1992). The protection that fruits and vegetables provide against diseases has been attributed to their high phytochemical content and corresponding antioxidant activity (Kahkonen et al., 1999 and Hollman et al., 1996). It is reported that increased consumption of fruits and vegetables elevate the antioxidant capacity of plasma in humans (Cao et al., 1998). Polyphenols, the most common antioxidants in plant materials, reduce oxidative stress due to their redox properties as hydrogen or electron donators (Rice-Evan et al., 1997).

Asparagus is one of the major sources of dietary polyphenols. Several studies measuring antioxidant capacities of fruits and vegetables using different methods have ranked asparagus first place among other vegetables (Tsushida et al., 1994; Vinson et al., 1998; Pellegrini et al., 2003). Prior and Wu (2007) reported that asparagus has a strong hydrophilic antioxidant capacity compared to other vegetables. However, a number of factors, including variety, growing conditions, location and season may affect the levels of phenolic compounds and thus, antioxidant activity.

Washington State is ranked second in total volume of asparagus produced in the United States. However, the harvest season of asparagus is short, from mid-April to mid-June in Washington. Harvest time is another parameter that may influence antioxidant activity.

Asparagus is a dioecious plant reproduced mainly by seed. Different approaches in breeding programs with the aim to increase yield and uniformity have led to the release of different kinds of materials since the beginning of the last century. A number of university breeding programs have spent intense effort towards developing new hybrids of asparagus that offer higher yields, increased rust resistance and tolerance to fusarium crown rot. During the last 40 years, emphasis has been given to all-male hybrids, which have better productivity and disease resistance.

Despite the continuous breeding studies that have been conducted with asparagus, there is limited information available on the differences in the antioxidant activity among varieties. Consumers are becoming increasingly concerned with healthy diets which affect their purchasing preference. Therefore, characterization of varietal differences will provide valuable information not only for consumer to make informed decisions but also for researchers to direct breeding studies towards those varieties having enhanced antioxidant value.

The purpose of this study was to evaluate the influence of variety and harvest date on polyphenolic composition and associated antioxidant activities of asparagus grown in Washington State. The study investigated the differences in the total phenolic, and rutin contents as well as antioxidant capacities of asparagus varieties as measured by spectrophotometric assays.

MATERIALS AND METHODS

Materials

Chemicals used in analyses: Folin-Ciocalteu reagent, rutin hydrate, 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH), Ethylenediaminetetraacetic acid (EDTA) disodium salt dihydrate and aluminum chloride hexahydrate were obtained from Sigma-Aldrich (St. Louis, MO). Sodium carbonate, sodium acetate anhydrous, glacial acetic acid, and methanol were purchased from J.T. Baker Inc (Phillipsburg, NJ)

Sampling procedure

Eight varieties of asparagus grown in Washington ('Jersey Knight', 'Jersey Deluxe', 'Jersey Supreme', 'Jersey Giant', 'Guelph Millenium', 'UC 157', 'Purple Passion' and 'Morehouse Select') were harvested at Schreiber Farms, and one variety, 'Syn 4', was harvested from an adjacent farm near Pasco, WA. Sampling was done at three different harvest dates during the 2006 season, at the time of optimum harvest maturity, as determined by the grower (April 25, May 17 and June 1). Each variety of asparagus was harvested from three different plots of the same field. Samples were picked up from field within an hour of being harvested, placed in an icebox, transported to the laboratory and kept at 4^{o}C overnight. Spears that had broken heads and diameters smaller than 0.5 inch were removed from selection pool. Spears were cut to 5 inches from the tip, weighed, chopped and ground for 1 min in a food processor (Cuisinart miniprep DLC1), placed in air-tight bags and stored frozen at -75^{o}C until analyses were carried out. All data collected for each asparagus variety at each harvest date were reported as means ± standard deviation (SD) for three replications.

Moisture content

The moisture content of asparagus varieties were measured according to AOAC method (AOAC, 1990). Approximately 2.0 grams of ground asparagus were weighed into aluminum dishes and dried at 70 ^{o}C for 24 hours. The moisture content was determined using the formula:

% moisture content = [(initial weight – final weight)/initial weight] * 100

Moisture content results were used to express the data of the study on a dry weight basis.

Extraction for TPH, Rutin content and Antioxidant Capacity (DPPH assay)

Two grams of samples were mixed with 20 mL deionized water and homogenized in an Omni-mix homogenizer (Ultra-Turrax T25, IKA Werke GmbH & Co., Germany) at speed 4.5 for 1 min. Homogenized samples were centrifuged using a Beckman J2-H2 centrifuge (Beckman, Palo Alto, CA) at 23700×g for 20 min at 4 ^{o}C. The supernatant phase was filtered through a Whatman No1 filter paper. Each sample set was extracted and immediately analyzed for TPH, rutin content and antioxidant capacity to avoid the loss of bioactive components.

Preparation of standard solutions

A stock rutin solution was prepared by dissolving 0.100 g of rutin in 250 mL methanol. Standard solutions were prepared by pipeting 0, 1, 2, 3, 4, 5, 8 and 10 mL of the stock solution into test tubes and bringing the volume to 10 mL with deionized water. Final concentrations of standard solutions were 0, 40, 80, 120, 160, 200, 320 and 400 mg/L.

Total phenolics

The total phenolic content of asparagus extracts were measured using Folin-Ciocalteu's reagent according to the method of Singleton and Rossi (1965). A volume of 0.3 mL extract was mixed with 6.0 mL deionized water and 0.5 mL of Folin-Ciocalteu reagent was added to each tube and agitated with a IKA mini vortexer MV1 (IKA Works, Wilmington, NC) for 5 s. After 30 sec to 8 min, 1.5 mL of 20% sodium carbonate solution was added and the volume brought to 10 mL with 1.7 mL deionized water and vortexed again for another 5 s. The mixture was allowed to stand at 20^{o}C for 2 hr. The absorbance was measured at 765 nm with an Ultraspec 4000 UV-visible spectrophotometer (Pharmacia Biotech, Cambridge, UK). Results were expressed as rutin equivalents in milligrams per gram of dry sample, using a standard curve generated with 80, 120, 160, 200, 320, and 400 mg/L of rutin.

Colorimetric determination of rutin content

Concentration of rutin was analyzed by the $AlCl_3$ spectrophotometric method described by Dame et al. (1959). A 2M acetate buffer solution, pH 5.4, was prepared by mixing 4.5 parts of 2M sodium acetate with 1 part of 2M glacial acetic acid. The buffer solution was mixed with an equal volume of 0.1M aluminum chloride to yield a final solution of pH 5.0. Rutin concentration was determined by mixing 0.5 mL of asparagus extract with 3.5 mL acetate/$AlCl_3$ buffer, agitating, allowing the sample to stand for 15 min, and then measuring the absorbance at 416 nm. From the same extract, a blank was prepared by mixing 0.5 mL extract with 3.5 mL of 2M acetate buffer (pH 5.0) without aluminum chloride, containing 0.5 mM disodium EDTA. The amount of rutin in mg/L

was calculated by subtracting the average absorbance value of the blank from the average absorbance value of the sample and comparing with a standard curve, prepared with 0, 40, 80, 120, 160 and 200 mg/L rutin. A volume of 0.5 mL of distilled water in 3.5 mL of the aluminum chloride/acetate buffer was used as reference to zero the spectrophotometer.

Antioxidant activity (DPPH radical scavenging assay)

The total antioxidant activity (TAA) of asparagus, determined by the activity of extracts to scavenge the DPPH radical, was measured according to the method of Brand-Willams et al. (1995). DPPH solution at a concentration of 6×10^{-5} mol/L was prepared by dissolving 0.012g DPPH reagent in 500 mL methanol. The DPPH solution was kept in the freezer in a tightly closed dark bottle until used. Twenty five microliter of asparagus extract was added to 0.975 mL of the DPPH solution and the decrease in absorbance was measured continuously at 515 nm during 120 min. The reaction reached a plateau in 90 min for all asparagus extracts and the standard as well. The same procedure was used for the determination of antiradical activity of extracts and absorbance was measured after 90 min. Concentrations of 0, 40, 80, 120, 160 and 200 mg/L of rutin were used to draw a standard curve and results were expressed as mg rutin equivalent/g dry asparagus.

Statistical analysis

All analyses were performed in triplicate and results were expressed as mean values ± standard deviations (Excel, Microsoft Inc., Redmond, WA). A randomized complete block design was used to evaluate significance differences among varieties and

harvest date. An ANOVA table including three factors and interaction was constructed using SAS 9.1.3 (SAS Institute Inc., Cary, NC). Tukey's all-pairwise comparison test was performed to determine significant differences of the data at $P \leq 0.05$. Correlation coefficient (R) was determined by regression analysis at the same confidence level using Minitab Statistical Software (Minitab Inc., State College, PA).

RESULTS AND DISCUSSION

Total phenolic content

The statistical analysis of data including the effects of variety, harvest date and sampling plot as well as the interaction between variety and harvest date is given in **Table 1**. A significant difference was found among varieties and harvest dates. The statistical differences were calculated using Tukey's all-pairwise comparison test. The total phenolic content of the nine asparagus varieties at three different harvest dates are ranked from largest to smallest in **Table 2.** Results ranged from 29.1 to 35.2 mg rutin equivalent/g of DW (2.59-3.23 mg rutin equivalent/g FW). 'Guelph Millenium' was found to have the largest phenolic content ($p \leq 0.05$) at 35.2 mg rutin equivalent/g of DW followed by 'Jersey Knight', 'Jersey Giant', 'Jersey Deluxe' and 'Jersey Supreme'. There was not a significant difference among 'Guelph Millenium', all Jersey varieties, 'Morehouse Select' and 'Syn 4' ($p \leq 0.05$). 'Purple Passion' and 'UC 157' showed the two smallest phenolic contents (29.1 and 30.2 mg rutin equivalent/g DW respectively) which are significantly different from 'Guelph Millenium' and 'Jersey Knight'. Therefore, two dioecious varieties ('UC 157' and 'Purple Passion'), which have both female and male

reproductive systems, exhibited small total phenolic content whereas all-male varieties ('Guelph Millenium', 'Jersey Knight', 'Jersey Giant', 'Jersey Deluxe', 'Jersey Supreme' 'Morehouse Select' and 'Syn 4') showed significantly greater values. Although total phenolic content demonstrated differences according to harvest date, there was not a chronological trend (**Figure 1**). Total phenolic contents decreased from the first (April 25) to the second harvest date (May 17) and increased again at the third harvest date (June 1). The mean daily temperature and solar radiation values for a 10-day period prior to each harvest date were obtained from the database of U.S. Bureau of Reclamation (USBR, 2007). Weather data indicates the observations for Lind, WA, which is 60 miles away from the asparagus field. As is seen in **Figure 4** the average air temperature significantly increased before the second harvest date and remained unchanged before the third harvest date. There was not a significant difference among solar radiation values. As concluded before by other researchers (Dame et al., 1957) harvest date does not directly affect phenol concentration in asparagus. However, growth rate and spear diameter may contribute to phenol accumulation. The total phenolic content of a spear may be a function of the time that it is above the ground prior to harvest. Thus a spear grown during warmer weather would have a faster rate of growth, have been above the ground for a shorter time, and have less total phenol content. Therefore, the decrease in the total phenol content at the second harvest date might be explained by the increase in the air temperature prior to harvest **(Figure 4)**. However total phenolic content rose again at the third harvest date while the average air temperature remained unchanged. Some other factors such as soil temperature, spear diameter of asparagus may also be affecting the

total phenol content. No interaction was found between variety and harvest date for total phenol content values.

The total phenolic content results presented here are very similar to those of Rodriguez et al. (2005). They found 1.62 and 2.25 mg rutin equivalent/g fresh weight (FW) for white and green asparagus 'Ramada' hybrids, respectively. Other cultivars of 'Grande' and native hybrids showed higher total phenol contents, which varied from 3.71 to 6.40 mg rutin equivalent/g FW asparagus. A possible reason for the relatively higher values of phenolic content in this study can be the differences in the extraction method. Rodriguez et al. (2005) used ethanol as extraction solvent while in the current study samples were extracted with water. The reason for selecting water was to estimate the phenolic compounds and antioxidant activity in asparagus juice. Vinson et al. (1998) studied the amount of phenolics in commonly consumed vegetables and reported that asparagus showed one of the greatest total phenolic contents on a dry weight basis after beet, red onion and broccoli. Another study investigating the differences in polyphenol concentration related to asparagus color revealed that total polyphenol content of green, purple and white asparagus were ranged 0.236-0.278, 0.269 and 0.142 mg quercetin equivalent/g FW, respectively (Maeda and Kakuta, 2005). Unlike the results given in **Table 2,** Maeda and Kakuta (2005) indicated that there was not a significant difference in total phenol content between purple and green asparagus. However, the results presented by Maede and Kakuta (2005) were based on a very limited number of samples (two green and one purple asparagus). The results of the present study included eight green and one purple asparagus varieties harvested from three different plots of the field at three different times during the season. Wang (2002) detected differences in the total phenolic

content of asparagus throughout the spear. The tip portion (21.6 mg rutin equivalent/g DW) of asparagus contained larger amount of total phenols than the middle and basal portions (17.8 and 13.5 mg rutin equivalent/g DW, respectively).

Rutin Content

The rutin contents of nine asparagus varieties, obtained by the $AlCl_3$ colorimetric method, are ranked from largest to smallest in **Table 3**. A significant difference was found among varieties and harvest dates for rutin content (**Table 1**). The results show that rutin content varies from 12.09 to 16.09 mg/g DW (1.08-1.43 mg/g FW) among the nine varieties. 'Guelph Millenium' was found to have the largest rutin content while 'Purple Passion' had the smallest. Varietal differences in the rutin content showed similar trends compared to the total phenolics. No significant difference was found between all-male varieties including 'Guelph Millenium', all Jersey varieties, 'Syn 4' and 'Morehouse Select' which also exhibited a parallel trend in total phenol contents. Resembling the total phenolic results, the other two varieties, 'UC 157' and 'Purple Passion' contained significantly lower levels of rutin content (12.32 and 12.09 mg/g DW respectively). A statistically significant correlation was found between total phenolics and rutin content as shown in **Figure 3** ($R=0.90$, $p\leq0.05$).

Previous studies also confirmed that rutin content of asparagus spears differed within varieties. Chin et al. (2002) examined the differences in rutin content of asparagus varieties developed in the Rutgers University breeding program and found a significant variation among different genotypes. They also reported variation throughout the asparagus spear; the tip portion of the spear had more rutin than the basal portion. They

reported that the average rutin level of the asparagus spear was 0.3 mg/g FW (Chin et al., 2002). Wang (2002) found that the tip portion (9.2 mg/g DW) contained 2.2 times more rutin than basal portion (4.1 mg/g DW). Stevenson (1950) reported 0.25-1 mg/g FW rutin in the edible parts of green asparagus. The tip of the spears had three to four times more rutin than the rest of the edible parts. Dame et al. (1950) found similar results in a comprehensive study of the composition of green asparagus stalk.

According to the results, the ratio of rutin content to total phenolics was approximately 45% **(Tables 2 and 3)**, which support the study of Makris and Rossiter (2001) who found that rutin was the predominant phenolic in methanolic extracts of asparagus. Dame et al. (1957) measured the total phenol and rutin contents of processed asparagus spears by Folin-Denis reagent and colorimetric $AlCl_3$ method, respectively. They reported that approximately 23% of the total phenol content of asparagus was contributed by rutin, which indicated the existence of other compounds that react with Folin reagent. Although ascorbic acid is not a phenol, it was found that it interferes with Folin reagent and contributes to 33% of the total phenolic content.

The results presented here are also in agreement with Wang et al. (2003) who worked on quantification of protodioscin and rutin in asparagus shoots by LC/MS and HPLC. They reported that asparagus contains rutin at levels of 0.3-0.6 mg/g FW. The level of rutin detected by the $AlCl_3$ colorimetric method in nine asparagus varieties varied between 1.08 and 1.43 mg/g FW. The higher results obtained may be due to differences in method used to measure rutin content. Kreft et al. (2002) reported that the $AlCl_3$ method gave 30% higher results than HPLC. One possible reason is that the $AlCl_3$ is a less selective method than HPLC and can react with other flavonoids in the samples.

Maeda and Kakuta (2005) reported that rutin content of purple spears (0.236 mg/g FW) was significantly higher than that of green spears (0.147 to 0.158 mg/g FW). This contradicting result is likely due to their small sample size, different extraction and analysis procedures and selecting different green varieties ('Welcome' and 'Gijnlim') for the study. They could not detect any rutin in white asparagus spears, which can be explained by the fact that exposure to light is essential for the rutin accumulation. Maeda and Kakuta (2005) also found a clear correlation between total phenol and rutin content.

The rutin content of asparagus varieties significantly increased at the end of the season. The average rutin content of asparagus in the third harvest date (June 1) was significantly higher than that of the first and second harvest dates (**Figure 4**). Generally, asparagus spear diameter decreases toward the end of the harvest season. Dame et al. (1957) reported an increase in the rutin content of asparagus with decrease in spear diameter. No interaction was found between variety and harvest date for rutin content values (**Table 1**).

Antioxidant capacity

The total antioxidant capacity was measured by the DPPH quenching method and results are presented as mg rutin/g of DW. Since rutin is the major flavonoid in asparagus spears, it could be reasonably expected that the extracts of asparagus demonstrate activities comparable to this flavonol. A significant difference was found among varieties and harvest dates for antioxidant capacity (**Table 1**). DPPH scavenging activities of nine varieties of asparagus are ranked from largest to smallest in **Table 4.** Results ranged from 10.70 mg rutin/g of DW for UC 157 to 15.30 mg rutin/g of DW for 'Jersey Knight'.

The antioxidant capacity of 'Jersey Knight' was followed by 'Guelph Millenium', 'Jersey Deluxe', 'Jersey Supreme', 'Morehouse Select', 'Jersey Giant' and 'Syn 4'. The two lowest values were obtained for 'Purple Passion' and 'UC 157' (11.55 and 10.70 mg rutin/g of DW, respectively). Significant varietal differences in antioxidant activity were observed between spears which showed highest ('Jersey Knight' and 'Guelph Millenium') and lowest ('Purple Passion' and 'UC 157') values according to the DPPH radical scavenging method. Rodriguez et al. (2005) also reported significant differences in quenching of the DPPH radical among asparagus varieties. Native hybrids from Huetor-Tajar exhibited significantly higher antiradical activity compared to 'Ramada' hybrids from Alcala del Rio. These results supported that sample cultivar had a great impact on antioxidant activity.

Tsushida et al. (1994) measured the main antioxidants in 43 vegetables by MS and found that rutin is the main antioxidant in asparagus. Makris and Rossiter (2001) pointed out that asparagus extracts demonstrated a similar β-carotene bleaching rate to that of rutin, indicating that rutin has an important effect on antioxidant activity. The relationship between rutin content and antioxidant activity of nine asparagus varieties was examined and as shown in **Figure 5** a significant correlation was observed ($R=0.74$, $p\leq0.05$). This result was also in agreement with the study of Maeda and Kakuta (2005) who observed a close relationship between rutin content and DPPH radical absorbing activity for asparagus varieties. However, for most of the varieties the levels of rutin content exceeded the level of DPPH radical activity expressed as mg rutin/DW asparagus (**Tables 3** and **4**). One possible reason for this discrepancy is the activity of polyphenol oxidase enzyme after extraction. Since asparagus samples were extracted using water, the

polyphenol oxidase enzyme remained active and might have caused a decrease in the antioxidant activity. Another possibility is that the $AlCl_3$ method overestimated the rutin content of asparagus. Kreft et al (2002) reported that $AlCl_3$ can react with other flavonoids in samples because of its non-selectivity.

A statistically significant correlation was also found between DPPH radical scavenging activity and total phenolics, as shown in **Figure 6** ($R=0.93$, $p\leq0.05$). These findings are consistent with results of Rodriguez et al. (2005), who found a significant relationship ($R=0.96$) between antiradical capacity and phenol content of several asparagus varieties. Tsushida et al. (1994) reported a high correlation ($R=0.77$) between polyphenol content and antioxidant activity. Sun et al (2005) stated a relation between total phenolic content and antioxidant activity of asparagus. Other studies also indicated similar significant relations for several fruits and vegetables (Velioglu et al, 1998; Chu et al., 2000; Cao et al., 1996; Ninfali et al., 2005). The Folin-Ciocalteu method for the determination of total phenolic content is based on redox properties of the compounds. Thus the values could partially express the antioxidant activity. This confirms a large correlation between the values of total phenolic content and the values of DPPH radical scavenging activity. However, the absolute differences between the two methods were high (**Tables 2** and **4**). The lower values of DPPH activity can be due to the fact that DPPH· is a long lived stable radical reacting only with very reactive phenolics and other antioxidants (Stratil et al., 2006).

Similar to total phenolic content values according to harvest dates, antioxidant activity showed a significant difference among harvest dates but there was not a specific trend (**Figure 7**). Antioxidant activity of asparagus varieties decreased from the first

(April 25) to the second harvest date (May 17) and increased at the third harvest date (June 1). The differences among harvest dates for antioxidant activities showed a similar pattern to that of total phenolic content. This similarity confirms the correlation between the two methods. No interaction was found between variety and harvest date for antioxidant capacities.

Several studies indicated that asparagus has a very strong antioxidant capacity among several vegetables. Pellegrini et al. (2003) ranked asparagus 1st in antioxidant capacity according to the TRAP assay (9.71 mmol Trolox/kg FW). Vinson et al. (1998) defined phenol antioxidant index (PAOXI) as the measure of the quality and quantity of antioxidants present in vegetables. It was calculated by dividing the total phenol concentration of the vegetable by the concentration of phenols in the extract that inhibit 50% of the oxidation of low density lipoproteins (IC_{50}). The vegetable with the largest dry weight PAOXI was asparagus among 23 vegetables. Halvorsen et al. (2002) reported the electron donating ability of Peruvian asparagus determined by the FRAP assay as 0.85 mmol/100 g FW. This value was larger than that of several vegetables such as celery, onion, broccoli, lettuce and cabbage. Wu et al. (2004) measured the lipophilic and hydrophilic antioxidant capacities of common foods in the U.S. using the $ORAC_{FL}$ assay. They reported that hydrophilic $ORAC_{FL}$ value of asparagus (29.15 μmol of TE/g) was much higher than lipophilic $ORAC_{FL}$ (1.02 μmol of TE/g). More than 96% of the total antioxidant capacity resulted from hydrophilic $ORAC_{FL}$. Foods were categorized into four groups ranked by their hydrophilic ORAC to total phenolics ratio and asparagus was placed in the highest group (Wu et al. 2004). Another study reported ORAC value of Argenteuil asparagus as 1288 μmol of Trolox equivalent/100 g (Ninfali et al., 2005).

Antioxidant activity of fruits and vegetables can change with processing conditions. Some researchers investigated the effect of processing on antioxidant activity of asparagus. Sun et al. (2007) studied antioxidant activity of asparagus after several thermal treatments and reported that microwave circulating water combination heating had advantage in maintaining the antioxidant activity. Nindo et al. (2003) worked on the effects of different drying technologies on antioxidants of asparagus. Their study indicated that Refractance Window drying and freeze drying methods enhanced the total antioxidant activity especially in the tip portion of asparagus spears.

The antioxidant capacities of varieties were also measured using the TEAC assay by another researcher in our laboratory. The data calculated as μM Trolox equivalent (TE)/g of DW asparagus are given in the **Appendix Table 1.** Results showed that hydrophilic fraction TEAC values were noticeably larger than the lipophilic fraction. To be able to compare the TEAC values with DPPH scavenging activity, a new standard curve was drawn for DPPH activity using Trolox standards and values were expressed as μM TE/g of DW (**Figure 8**). Both of the antioxidant capacity assays exhibited consistent results. According to TEAC data, the highest activity was obtained for 'Guelph Millenium' with 69.10 μM TE/g of DW followed by 'Jersey Knight', 'Jersey Supreme', 'Jersey Giant', 'Jersey Deluxe' and 'Morehouse Select'. No significant difference was found among those varieties. 'Syn 4' exhibited significantly lower activities while 'UC 157' and 'Purple Passion' showed the lowest (49.38 and 49.37 μM TE/g of DW, respectively).

CONCLUSION

This study suggests that asparagus is a rich source of phenolic compounds. Asparagus varieties were found to possess high total phenolic content and antioxidant activity. Rutin was found to be the major phenol in asparagus. Rutin as well as its aglycones quercetin are important in the human diet. Quercetin and glycosides have been reported to modulate a number of biochemical and pharmacological activities such as scavenging free radicals, chelating metal ions, helping immune and inflammatory cell functions and anticarcinogenic effects (Middleton and Kandaswami, 1992). Antioxidant activity of asparagus is positively correlated with total phenolic and rutin content ($p \leq 0.05$), suggesting that phenols are mainly responsible for antioxidant activity. It is noteworthy that all-male hybrid varieties, 'Guelph Millenium', 'Jersey Knight', 'Jersey Deluxe', 'Jersey Supreme', 'Jersey Giant', 'Morehouse Select', and 'Syn 4' showed significantly greater total phenolic and rutin contents as well as greater antioxidant activity than dioecious varieties, 'Purple Passion' and 'UC 157'. Results were also affected by harvest date. While rutin content significantly increased at the end of the season, total phenol and antioxidant capacity did not exhibit a specific trend.

The results of the study revealed that the phenol content and associated antioxidant activity demonstrate differences due to genetic variation and harvest dates. Knowledge of specific differences in the phenolic profile and antioxidant activity among asparagus varieties may provide additional value to breeders in selecting the hybrids with specific health benefits.

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Table 1. Statistical analysis for total phenolics, rutin content and antioxidant activity

	Total Phenolics		Rutin Content		Antioxidant activity	
Source of variation	F value	*p* value	F value	*p* value	F value	*p* value
Variety	6.67	<0.0001*	5.81	<0.0001*	7.16	<0.0001*
Harvest date	14.25	<0.0001*	15.46	<0.0001*	24.53	<0.0001*
Sampling plot	0.81	0.4511	0.49	0.6159	4.80	0.0124*
Variety*harvest date (interaction)	1.47	0.1493	1.82	0.0534	1.27	0.2505

***** *p* values ≤ 0.05 show statistically significant difference.

Table 2. Total phenolic contents of nine asparagus varieties at three different harvest dates

Variety	1st harvest	2nd harvest	3rd harvest	Mean ± std[1]
Guelph Millenium	35.56 ± 0.71	33.23 ± 1.93	36.69 ± 4.87	**35.16 ± 1.76[a]**
Jersey Knight	34.72 ± 2.85	32.99 ± 2.66	36.92 ± 1.30	**34.88 ± 1.97[a]**
Jersey Giant	33.69 ± 1.63	32.50 ± 1.21	35.33 ± 1.60	**33.84 ± 1.42[ab]**
Jersey Deluxe	35.84 ± 2.44	29.54 ± 3.93	35.92 ± 1.53	**33.77 ± 3.66[ab]**
Jersey Supreme	36.41 ± 1.38	30.17 ± 2.44	34.10 ± 1.59	**33.56 ± 3.15[ab]**
Morehouse Select	33.74 ± 3.43	31.72 ± 2.55	33.60 ± 1.17	**33.02 ± 1.13[ab]**
Syn 4	29.93 ± 1.02	32.47 ± 0.49	34.36 ± 0.88	**32.25 ± 2.22[abc]**
UC 157	30.50 ± 2.09	29.37 ± 0.61	30.69 ± 2.49	**30.19 ± 0.71[bc]**
Purple Passion	27.47 ± 2.61	27.27 ± 2.86	32.53 ± 2.97	**29.09 ± 2.98[c]**

Values are expressed as mg rutin equivalent/g dry weight asparagus and represent means ± SD (n=3)

[1]Means with different letters indicate significant differences ($P \leq 0.05$) by Tukey's test

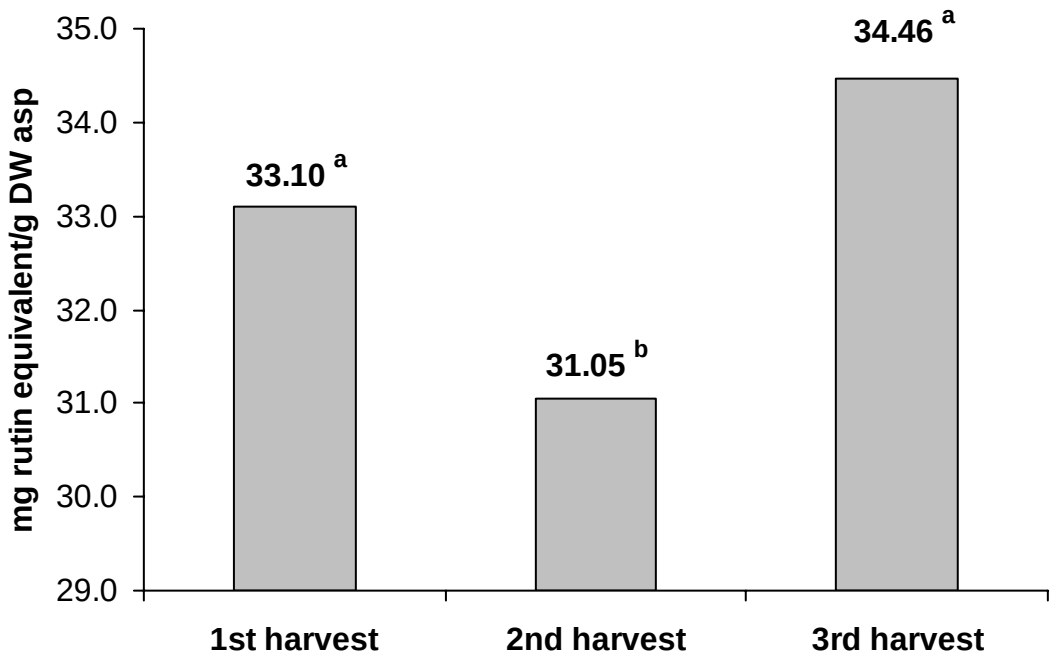

Figure 1. Total phenolic content of asparagus at different harvest dates

* The data are means of nine varieties of asparagus

* Means with different letters indicate significant differences ($P \leq 0.05$) by Tukey's test

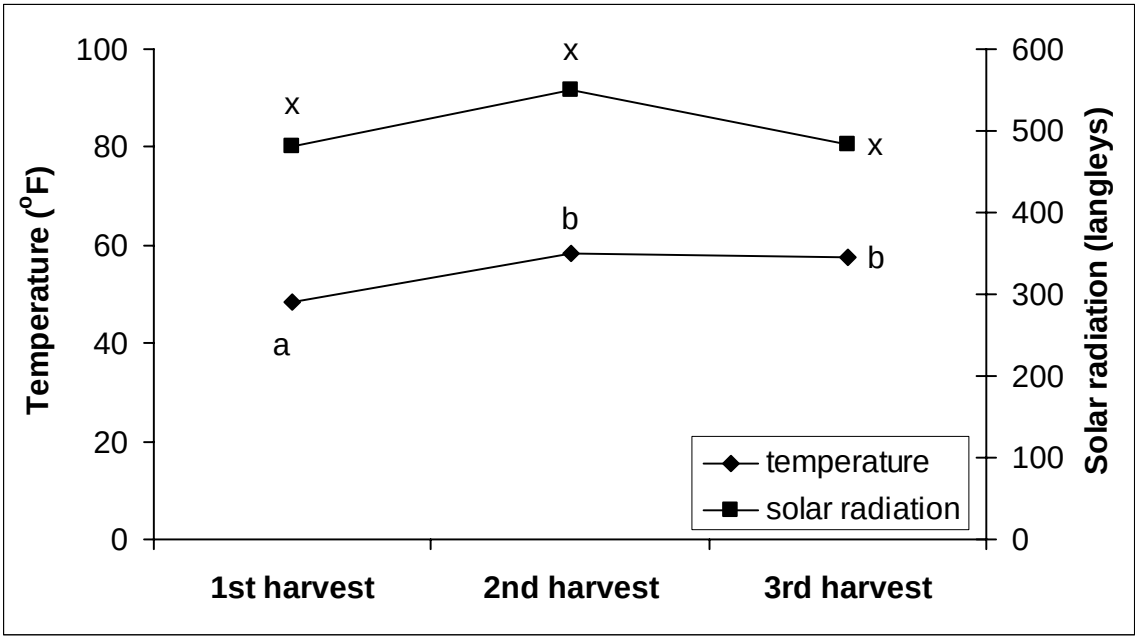

Figure 2. Temperature and solar radiation measurements before each harvest date

* Left hand side y axis shows the average of daily air temperature (°F) for a 10 day period before each harvest date.

* Right hand side y axis shows the average of daily solar radiation (langleys) for a 10 day period before each harvest date.

* Temperature and solar radiation values were measured in Lind, WA (USBR, 2007).

* Means with different letters indicate significant differences ($P \leq 0.05$) by Tukey's test

Table 3. Rutin contents of nine asparagus varieties at three different harvest dates.

Variety	1st harvest	2nd harvest	3rd harvest	Mean ± std[1]
Guelph Millenium	16.05 ± 0.17	14.00 ± 1.43	18.21 ± 3.91	**16.09 ± 2.11[a]**
Jersey Deluxe	15.89 ± 2.16	13.69 ± 2.52	17.53 ± 1.06	**15.70 ± 1.93[a]**
Syn 4	13.11 ± 1.29	17.08 ± 1.29	16.81 ± 0.92	**15.67 ± 2.22[a]**
Jersey Giant	15.45 ± 1.25	14.45 ± 1.20	16.81 ± 1.29	**15.57 ± 1.18[a]**
Jersey Knight	15.86 ± 0.67	13.53 ± 1.03	16.24 ± 1.45	**15.21 ± 1.47[a]**
Jersey Supreme	16.21 ± 0.67	13.61 ± 1.42	15.42 ± 1.42	**15.08 ± 1.33[ab]**
Morehouse Select	14.17 ± 1.41	13.89 ± 2.11	15.72 ± 1.88	**14.59 ± 0.99[abc]**
UC 157	12.62 ± 0.87	11.56 ± 0.66	12.77 ± 1.95	**12.32 ± 0.66[bc]**
Purple Passion	10.69 ± 1.84	9.44 ± 2.66	16.14 ± 3.38	**12.09 ± 3.56[c]**

Values are expressed as mg rutin/g dry weight asparagus and represent means ± SD (n=3)

[1]Means with different letters indicate significant differences ($P \leq 0.05$) by Tukey's test

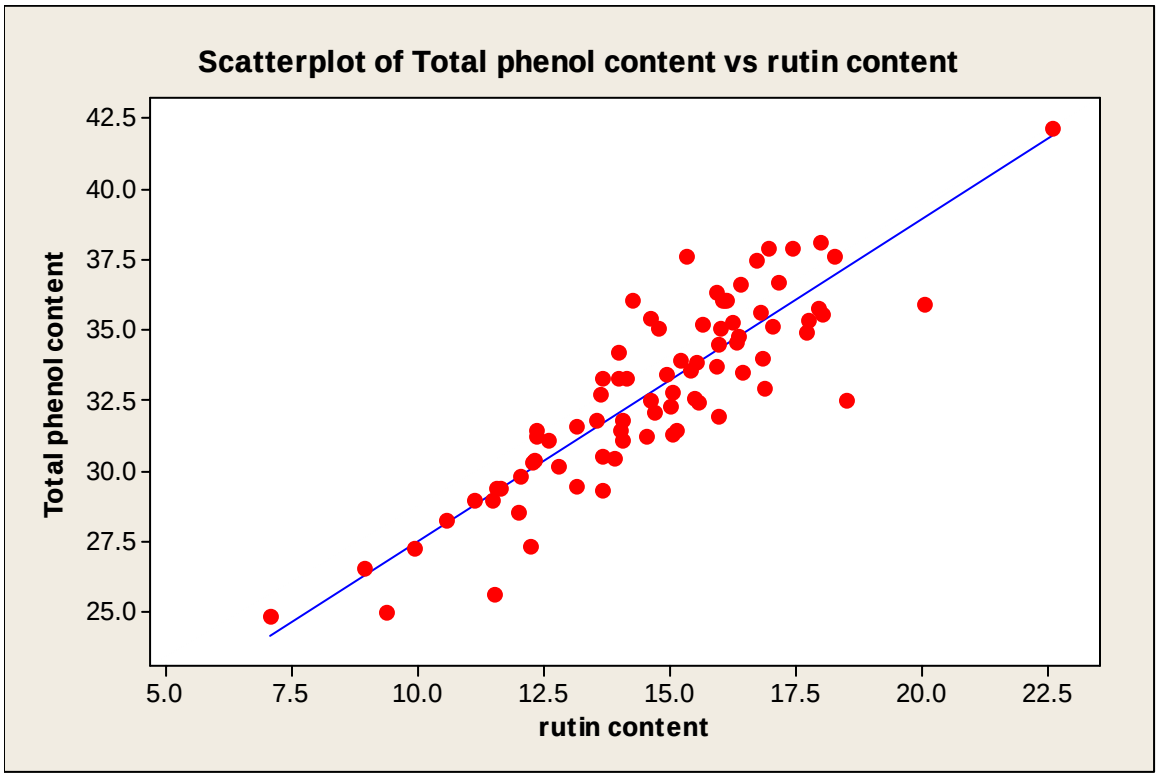

Figure 3. Relationship between total phenolic and rutin contents of asparagus (R=0.90)

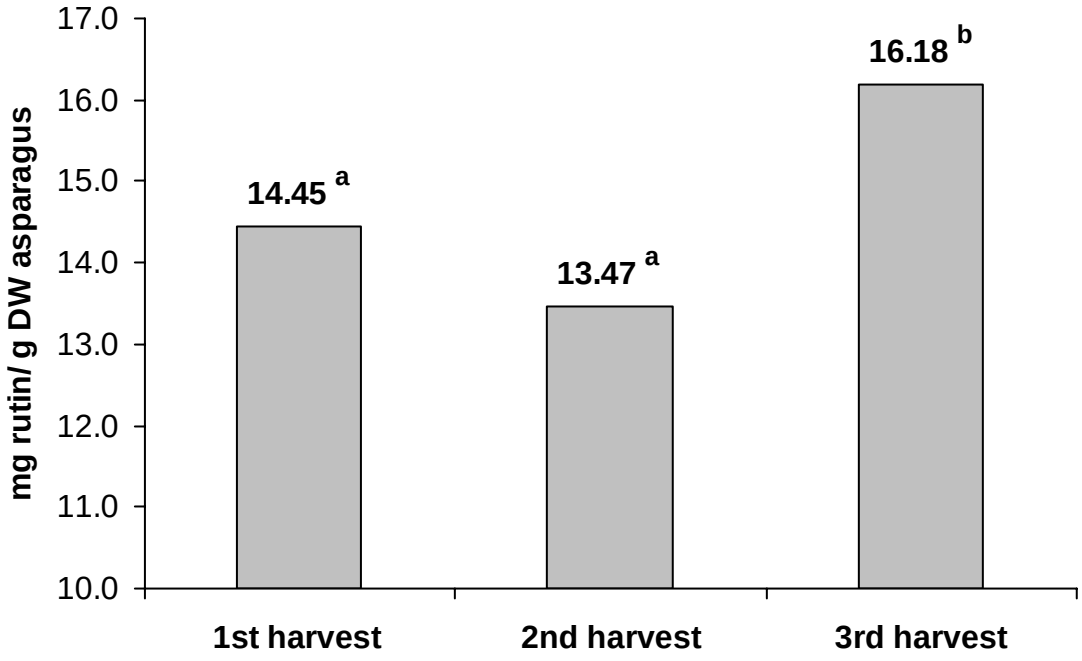

Figure 4. Rutin content of asparagus at different harvest dates.

* The data are means of nine varieties of asparagus

* Means with different letters indicate significant differences ($P \leq 0.05$) by Tukey's test

Table 4. Antioxidant activities of nine asparagus varieties measured by quenching of the DPPH radical at three different harvest dates

Variety	1st harvest	2nd harvest	3rd harvest	Mean ± std[1]
Jersey Knight	16.0 ± 1.4	12.6 ± 3.7	17.3 ± 0.8	**15.30 ± 2.39[a]**
Guelph Millenium	13.9 ± 1.2	13.0 ± 1.5	16.1 ± 2.7	**14.32 ± 1.56[ab]**
Jersey Deluxe	15.6 ± 1.0	10.4 ± 0.8	15.2 ± 1.5	**13.74 ± 2.88[abc]**
Jersey Supreme	13.9 ± 1.7	11.1 ± 1.6	14.2 ± 1.4	**13.04 ± 1.71[abc]**
Morehouse Select	14.8 ± 1.6	11.2 ± 1.2	12.9 ± 1.3	**12.98 ± 1.83[bc]**
Jersey Giant	12.9 ± 1.4	11.5 ± 1.2	14.4 ± 0.4	**12.96 ± 1.46[bc]**
Syn 4	12.7 ± 0.6	11.3 ± 1.1	12.7 ± 0.8	**12.23 ± 0.81[bc]**
Purple Passion	11.4 ± 1.8	10.5 ± 2.7	12.8 ± 1.1	**11.55 ± 1.13[c]**
UC 157	10.7 ± 1.5	10.1 ± 1.0	11.4 ± 1.9	**10.70 ± 0.65[c]**

Values are expressed as mg rutin equivalent/g dry weight asparagus and represent means ± SD (n=3)

[1]Means with different letters indicate significant differences ($P \leq 0.05$) by Tukey's test

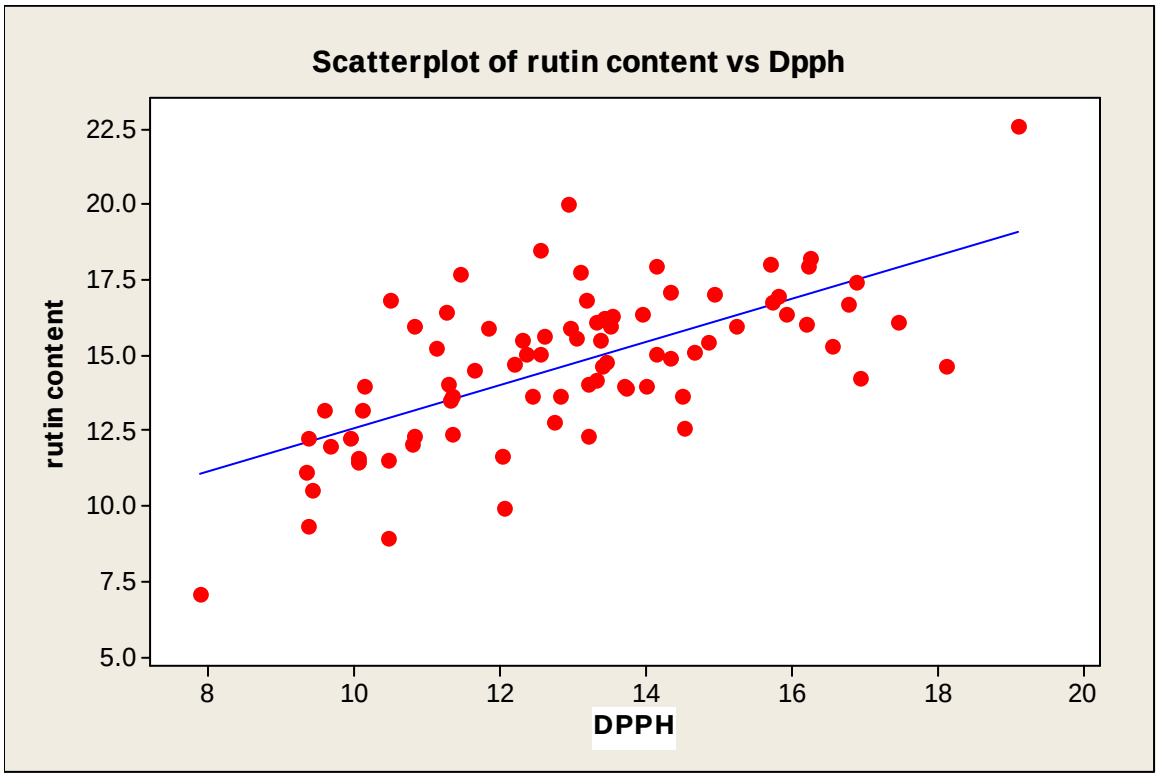

Figure 5. Relationship between rutin content and antioxidant activity (DPPH radical scavenging activity) of asparagus (R=0.74)

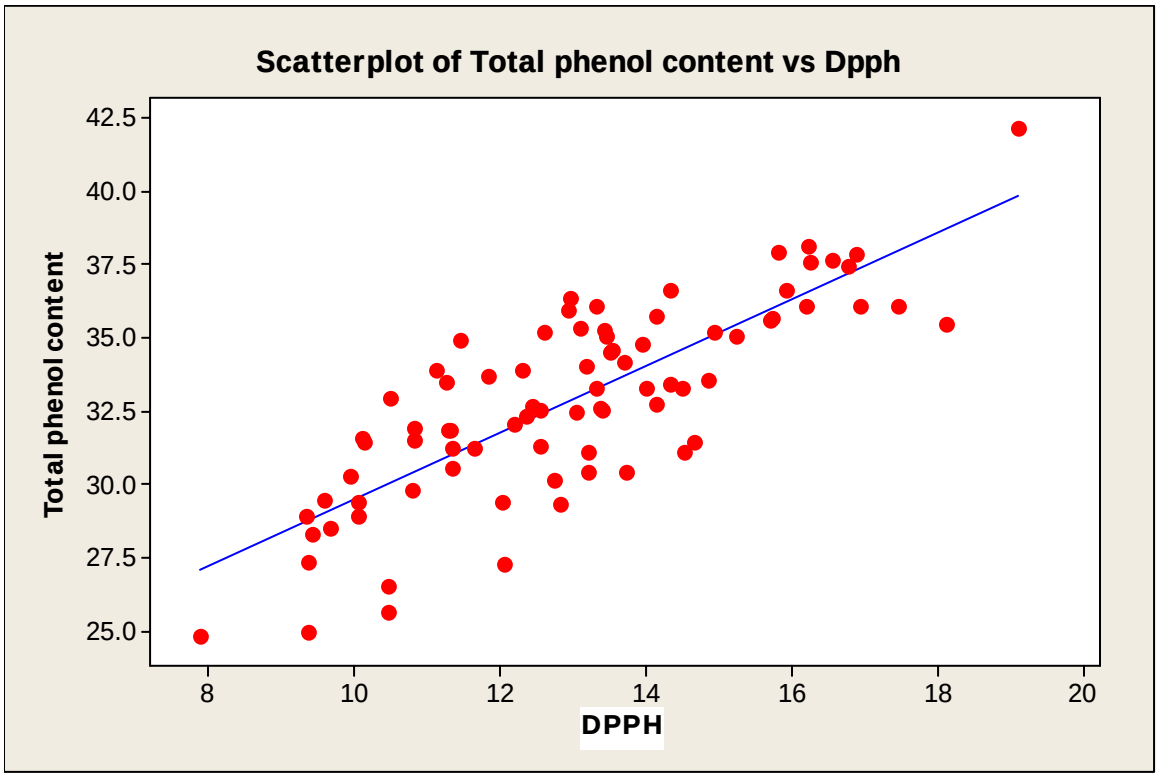

Figure 6. Relationship between total phenolic content and antioxidant activity (DPPH radical scavenging activity) of asparagus (R=0.93)

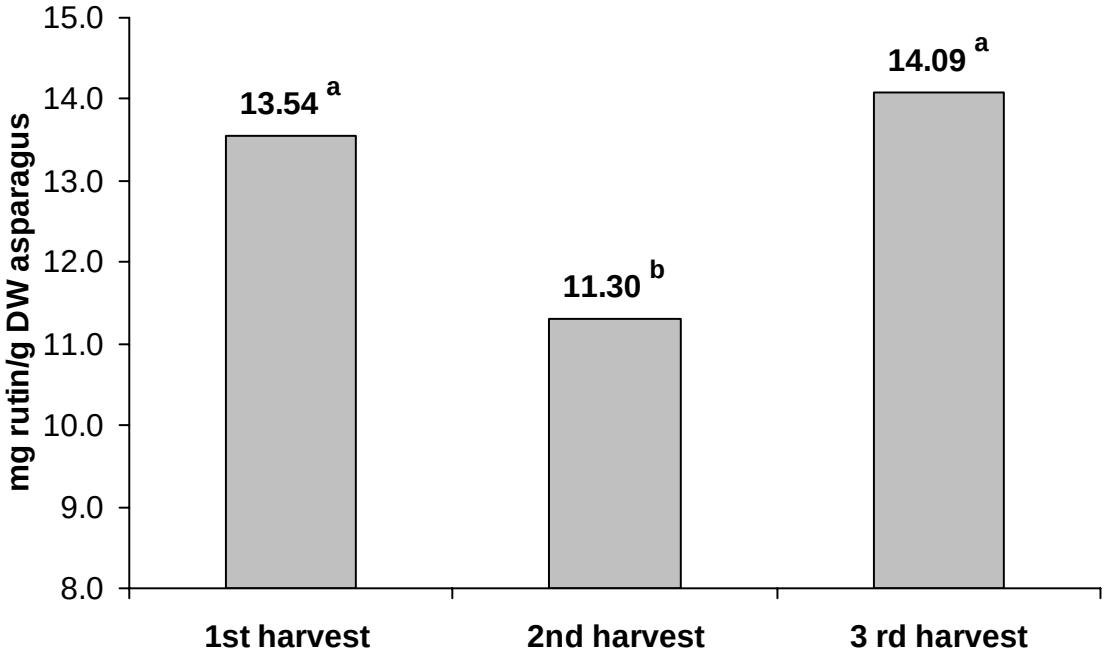

Figure 7. DPPH radical scavenging activity of asparagus at different harvest dates

* The data are means of nine varieties of asparagus

* Means with different letters indicate significant differences ($P \leq 0.05$) by Tukey's test

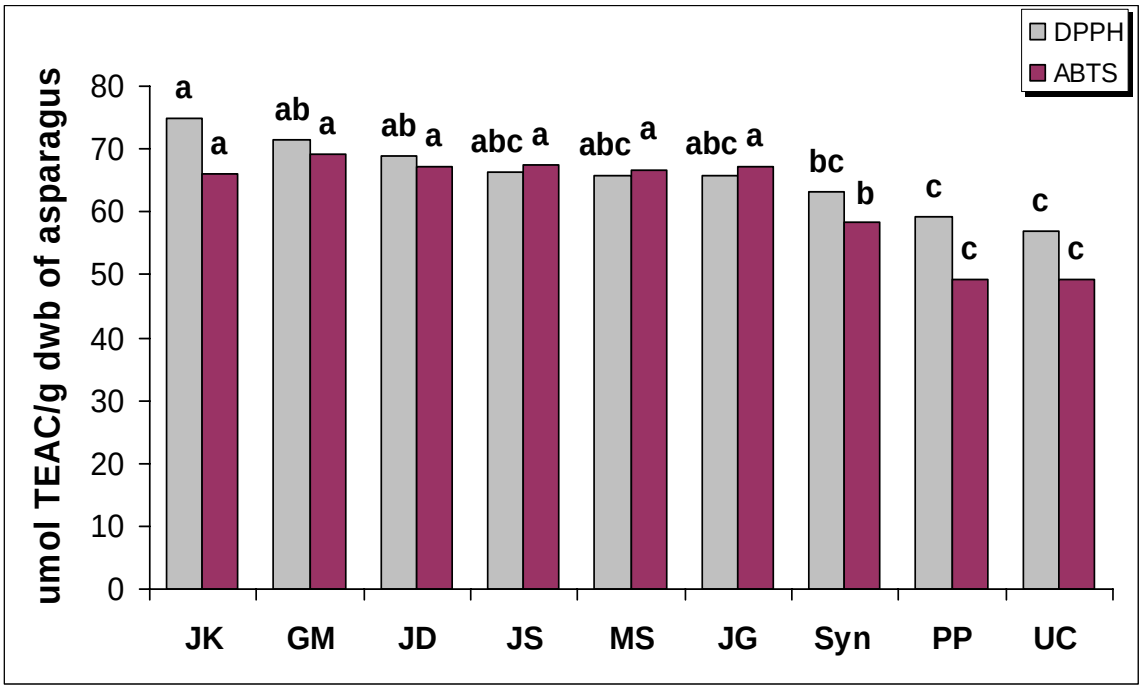

Figure 8. Comparison of ABTS and DPPH radical scavenging activities for nine varieties of asparagus.

* Values are expressed as umol TEAC/g dry weight asparagus and represent means of three harvest date. Means with different letters indicate significant differences ($P \leq 0.05$) by Tukey's test.

Abbreviations used for asparagus varieties:

JK: 'Jersey Knight'
GM: 'Guelph Millenium'
JD: 'Jersey Deluxe'
JS: 'Jersey Supreme'
MS: 'Morehouse Select'
JG: 'Jersey Giant'
Syn: 'Syn 4'
PP: 'Purple Passion'
UC: 'UC 157'

CHAPTER TWO

DETERMINATION OF PHENOLIC CONSTITUENTS AND ANTIRADICAL COMPOUNDS OF ASPARAGUS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

ABSTRACT

Nine varieties of asparagus were analyzed for their phenolic constituents using a reversed-phase high performance liquid chromatography (HPLC) coupled with a diode array detector. Rutin was identified as the major phenolic compound, followed by chlorogenic acid, in all varieties. An HPLC-DPPH method was also used to determine radical scavenging components. Antiradical activity was measured by the decrease in the chromatographic peak intensity, 60 min after the addition of the DPPH. The method was applied to a green ('Guelph Millenium') and a purple ('Purple Passion') asparagus variety to detect and identify the antiradical compounds in different colors of asparagus. The HPLC analysis revealed rutin is the strongest radical scavenging component. After the addition of the DPPH solution, the peaks corresponding to rutin and chlorogenic acid decreased by 93% and 72% of the initial peak intensity, respectively. Purple asparagus had a unique peak different than green asparagus varieties. This unknown compound did not possess antiradical activity. Total anthocyanin contents of asparagus varieties were measured spectrophotometrically. 'Purple Passion' contained the largest total anthocyanin content (1.08 mg/g dwb) among the varieties tested, and it was 4 times greater than 'UC 157' which had the smallest anthocyanin content (0.21 mg/g dwb).

KEYWORDS: HPLC, phenolic compounds, asparagus, anthocyanins, DPPH, antiradical

INTRODUCTION

High performance liquid chromatographic (HPLC) methods are commonly used for separation and quantification of phenolic compounds. Various stationary and mobile phases are available for the analysis of each type of flavonoid. In particular, the development of reversed phase columns has greatly improved the separation performance of phenolics by HPLC (Macheix, et al., 1990). Several reviews on the application of HPLC for the analysis of phenolics have been published (Merken and Beecher, 2000; Robards and Antolovitch, 1997). Since the standard compounds for many flavonoid glycosides are not available, hydrolysis of glycosides into aglycones has become an accepted practice before HPLC analysis (Mattila et al., 2000; Cai et al., 2003). Detection of individual flavonoid compounds by HPLC methods has mostly been based on UV-Vis absorption. Use of diode array detection (DAD), which enables the collection of on-line spectrum and simultaneous quantification at several wavelengths, has become popular for phenolic compound analysis (Hertog et. al, 1992). Diode array and electrochemical detection produce additional data which can be used in identifying the peaks.

Some studies have previously reported the phenolic compounds that exist in asparagus. A study investigating the effects of processing on flavonols and antioxidants reported that rutin is the predominant flavonoid accompanied by five other undefined minor phenolics (Makris and Rossiter, 2001). Most recently, Wang et al. (2003) used LC/MS to quantitate the sterol protodioscin and rutin in asparagus. Sakakibara, et al (2003) identified some other simple phenols such as chlorogenic acid, caffeic acid and cinnamic acid in addition to rutin. Most of these studies have been carried out in order to

identify the main phenolic compounds in asparagus. However, information concerning the correlation of phenolic compounds with antioxidant activity is not available.

In recent years, several on-line HPLC methods coupled to a radical scavenging reaction have been developed for the detection of antioxidant compounds. (Bandoniene and Murkovic, 2002a,b; Masuda et al., 2003**;** Nuengchamnong et al., 2005). The determination of antioxidative constituents is based on corresponding decrease or loss of peaks on HPLC chromatograms by the reaction with the DPPH radical. The greatest benefit of this technique is that it allows for a rapid and selective detection of radical scavenging substances with a minimum of sample preparation.

Anthocyanins are flavonoids implicated as antioxidants and have been isolated from asparagus (Flores et al., 2005). Anthocyanins are responsible for the pink color of some varieties. After harvesting, anthocyanin synthesis continues in asparagus tissue and pink coloration occurs during storage (Siomos et al. 2000).

The second part of the present study has focused on identifying polyphenolic profile of asparagus after a multi-step extraction process. Flavonoid extracts were analyzed using HPLC equipped with a photodiode array detector (DAD). DAD was performed simultaneously in the system, thus aiding the identification of individual flavonoids. Combining the spectrum and elution time information resulted in specific, selective, and precise flavonoid analysis. Anthocyanin extracts were examined by a pH difference method using a spectrophotometer. In addition, a screening method was developed for determination of radical scavenging compounds in asparagus, based on a pre-column reaction of the asparagus extracts with the DPPH radical.

MATERIALS AND METHODS

Materials

Formic acid, phosphoric acid, hydrochloric acid, diethyl ether, ammonium sulfate, HPLC grade methanol and HPLC grade ethyl acetate were purchased from J.T. Baker Inc (Phillipsburg, NJ). Rutin hydrate, chlorogenic acid and metaphosphoric acid were obtained from Sigma-Aldrich (St. Louis, MO).

Ethanol (95%) was purchased from Washington State University Central Store.

HPLC standards: chlorogenic acid, ferulic acid, caffeic acid, cinnamic acid, ellagic acid, coumarin, catechin, epicatechin, epigallocatechin, gallocatechin, epigallocatechin gallate, quercetin, rutin, fisetin, kaempferol, myricetin, gossypetin, taxifolin, hesperetin, naringenin, eriodictyol, homoeriodictyol, luteolin, apigenin, stilbene, pterostilbene, pinosylvin, phloretin, daidzein and cyanidin chloride were obtained from commercial sources (Indofine Chemical Company, Inc., Belle Mead, NJ; Sigma-Aldrich Corporation, St. Louis, MO).

Sampling procedure

Eight varieties of asparagus grown in Washington ('Jersey Knight', 'Jersey Deluxe', 'Jersey Supreme', 'Jersey Giant', 'Guelph Millenium', 'UC 157', 'Purple Passion' and 'Morehouse Select') were harvested at Schreiber Farms, and one variety, 'Syn 4', was harvested from an adjacent farm near Pasco, WA. Sampling was done at three different harvest dates during the 2006 season, at the time of optimum harvest maturity, as determined by the grower (April 25, May 17 and June 1). Each variety of asparagus was harvested from three different plots of the same field. Samples were picked up from field

within an hour of being harvested, placed in an icebox, transported to the laboratory and kept at 4^{o}C overnight. Spears that had broken heads and diameters smaller than 0.5 inch were removed from selection pool. Spears were cut to 5 inches from the tip, weighed, chopped and ground for 1 min in a food processor (Cuisinart miniprep DLC1), placed in air-tight bags and stored frozen at -75^{o}C until analyses were carried out. All data collected for each asparagus variety at each harvest date were reported as means ± standard deviation (SD) for three replications.

Moisture content

The moisture content of asparagus varieties were measured according to AOAC method (AOAC, 1990). Approximately 2.0 grams of ground asparagus were weighed into aluminum dishes and dried at 70 ^{o}C for 24 hours. The moisture content was determined using the formula:

% moisture content = [(initial weight – final weight)/initial weight] * 100

Moisture content results were used to express the data of the study on a dry basis.

Extraction of Phenolics and Anthocyanins

Asparagus samples were extracted using a modified procedure of Miller et al. (1998). Asparagus samples were removed from –75^{o}C and about 5 g of sample was weighed into 50 mL Pyrex test tubes and 10 mL of an acidified extraction solution (80% ethanol, 1% formic acid) was added. The samples were further ground with an Omni-mix homogenizer until tissue was thoroughly macerated. Samples were transferred to the centrifuge tubes after an addition of 10 more mL of the same solution and centrifuged

using a Beckman J2-H2 centrifuge (Beckman, Palo Alto, CA) at 9620 x g for 30 min. The supernatants were poured over a Whatman No. 4 paper into a round bottom flask. An additional extraction solution of 15 mL (50% ethanol, 1% formic acid) was then poured into flask through the filter. Samples were dried to 10 mL at 55^{o}C in a rotary evaporator under vacuum (Buchler Instruments, Fort Lee, NJ) and transferred into 50 mL Pyrex test tubes.

To remove lipids and non-polar pigments such as chlorophylls and carotenoids, diethyl ether was applied to the samples 3 times. Each time 5 mL of diethyl ether was added to the extract and test tube was inverted using a rubber stopper. The solution was allowed to sit for 5 min for separation of two fractions. The top fraction (ether phase) was removed using a glass pipette and discarded. The test tube was left in the hood for 30 min to allow the remaining ether to evaporate before proceeding.

The next stage includes purification of phenolic compounds by partitioning of depigmented aqueous extract with ethyl acetate. Phenolic extraction solution of 2.5 mL (20% ammonium sulfate, 20% ethanol, 2% metaphosphoric acid) was added to previous extract. Samples were solvent partitioned 3 times with 5 mL HPLC grade ethyl acetate. Each time 5 mL of ethyl acetate was added to the extract and test tube was mixed by inverting. The solution was allowed to sit for 5 min for separation of the two phases. The top fraction (ethyl acetate phase) was removed using a glass pipette and pooled into a 150 mL vacuum flask. The remaining aqueous fraction (anthocyanin phase) was collected and kept frozen at -75^{o}C until the anthocyanin analysis. The ethyl acetate was evaporated under vacuum at 60^{o}C leaving a semi-yellow residue. The dried phenolic residue in the vacuum flask was dissolved in 2 mL of HPLC grade methanol and centrifuged at 8000

rpm (VWR International, Galaxy 14D, West Chester, PA) for 5 min. Samples (2 mL) were syringe filtered through 0.45 µm Millipore glass fiber prefilters (Millipore Corp., Bedford, MA) into 2 mL borosilicate glass vials with a teflon septum (VWR International, West Chester, PA) and stored at -75^{o}C until analysis.

Total Anthocyanin Content Analysis

Total anthocyanins were determined using a modification of the method of Fuleki and Francis (1968) after samples were extracted as describe above. An aliquot of 1.0 mL extract was diluted with 4.0 mL of ethanol-1.5 *N* HCl (85:15), and then placed into darkness for 2 hours. The pH of each sample was 1.0 ($\pm$0.1). The absorbance was then measured at 535 nm with an Ultraspec 4000 UV-visible spectrophotometer (Pharmacia Biotech, Cambridge, UK). Anthocyanin content was calculated as mg total anthocyanins per g of dry weight asparagus with the appropriate weight, volume, dilution factors and extinction value. The total optical density (TOD) is calculated first then it is transformed to mgs of anthocyanin with the aid of the extinction coefficient (E value). The E value of cranberry anthocyanins of 98.2 (mg/ml)cm^{-1}, proposed by Fuleki and Francis (1968) was used in the calculation.

The TOD for 1 ml extract is calculated using the following formula:

TOD = OD x DV x VF

OD = the absorbance at 535 nm

DV = the volume in mLs of the diluted extract prepared for the OD measurement

VF = the volume of the sample before dilution

Total anthocyanin (T Acy) in mgs per g dwb was calculated using the equation:

T Acy = [TOD per mL x TEV/ W]/E

TEV = total extract volume

W = the dry weight of the sample

E = extinction coefficient (*98.2*)

Analysis of Phenolic Compounds

The HPLC system consisted of an Agilent 1100 series pumping system, autosampler, column oven and an internal DAD (Palo Alto, CA) equipped with a C-18 Zorbax-SB column (150 x 4.6 mm i.d., 5 μM particle size) and guard column (12.5 x 4.6 mm i.d., 5 μM particle size) (Agilent Technologies Inc, New Castle, DE). Phenolic extracts (10 μl) were injected and subjected to a gradient elution program consisting of a mobile phase: 0.5% phosphoric acid in water (solvent A) and 100% methanol (solvent B). The program used a linear gradient starting from 90% A and 10% B, increased to 30% A and 70% B over 42 min, and reached to 100% B at 47 min. The column was cleaned with 100% methanol (B) for 13 min, and re-equilibrated to 90% A and 10% B for 5 minutes. Flow rate was set at 1.0 ml/min and column temperature was held at 40^{o}C.

The extracts of nine asparagus varieties were separated by HPLC and the DAD spectra of different peaks were recorded. Identification of the peaks in the chromatograms was performed by comparing the HPLC signal response and absorbance spectrum of the sample peak with the response and spectrum of pure standards under the same elution conditions. A number of standards including phenolic acids (chlorogenic acid, ferulic acid, caffeic acid, cinnamic acid, ellagic acid, coumarin), flavan-3-ols (catechin, epicatechin, epigallocatechin, gallocatechin, epigallocatechin gallate),

flavonols (quercetin, rutin, fisetin, kaempferol, myricetin, gossypetin), flavanones (taxifolin, hesperetin, naringenin, eriodictyol, homoeriodictyol), flavones (luteolin, apigenin), stilbenes (stilbene, pterostilbene, pinosylvin), chalcone (phloretin), isoflavone (daidzein), and anthocyanin (cyanidin chloride) were used to identify different phenolics. Relative quantifications of flavonoid/phenolic compounds were calculated by preparing a standard curve with concentrations of 0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL of selected phenolic standards. The area under the peaks was plotted against the concentration of phenolic standard. DAD signal for detection was set to 254 nm.

HPLC-DPPH analysis of radical scavenging compounds

An HPLC method coupled with the DPPH radical scavenging assay was used with some modifications for a rapid detection of antiradical compounds (Masuda et al., 2003). Phenolic extract (100 μL) was mixed with 5 mM DPPH (100 μL) solution in methanol. The mixture was stirred and allowed to stand for 60 min at 25^{o}C. The mixture (10 μL) was injected into the HPLC and analyzed under the same conditions described above. HPLC data obtained by direct injection of each extract were used as reference. The disappearance or decrease in the peak intensity induced by the addition of the DPPH radical is monitored by the HPLC chromatograms. DPPH solution in methanol (10 μL) was injected separately as a blank.

Statistical analysis

All analyses were performed in triplicate and results were expressed as mean values ± standard deviations (Excel, Microsoft Inc., Redmond, WA). A randomized complete block design was used to evaluate significance differences among varieties and harvest date. An ANOVA table including three factors and interaction was constructed using SAS 9.1.3 (SAS Institute Inc., Cary, NC). Tukey's all-pairwise comparison test was performed to determine significant differences of the data at $P \leq 0.05$. Correlation coefficient (R) was determined by regression analysis at the same confidence level using Minitab Statistical Software (Minitab Inc., State College, PA).

RESULTS AND DISCUSSION

Total Anthocyanins

The statistical analysis of data including the effects of variety, harvest date and sampling plot as well as the interaction between variety and harvest date is given in **Table 1**. A significant difference was found among varieties and harvest dates. The statistical differences were further calculated using Tukey's all-pairwise comparison test. The total anthocyanin contents of nine asparagus varieties are ranked from largest to smallest in **Table 2**. Results ranged from 0.21 to 1.08 mg/g of DW (19.91-96.75 μg/g FW) among the nine varieties. 'Purple Passion' exhibited the largest anthocyanin content (1.08 mg/g DW) as can be predicted by its purple color. Jersey varieties and 'Syn 4' are known to have green spears with purple bracts. These varieties showed the second largest

values after ‘Purple Passion’ and there was no significant difference ($p>0.05$) among their anthocyanin contents.

Two open pollinated varieties, ‘UC 157’ and ‘Purple Passion’ were detected as outliers. These two varieties exhibited similar rutin content and antioxidant activity in the first part of the study. However, their anthocyanin contents were significantly different. ‘UC 157’ possessed the smallest levels of anthocyanins (0.26 mg/g DW), while ‘Purple Passion’ showed the greatest. Even though ‘Purple Passion’ contained greater amounts of anthocyanins, this was not reflected in its DPPH radical scavenging capacity. The antioxidant activity of ‘Purple Passion’ was not significantly different than ‘UC 157’. These finding suggested that asparagus anthocyanins are not as powerful antioxidants as rutin and other phenolics. **Figure 1** illustrates the chemical structure of the major anthocyanin (cyanidin-3-glucoside) and the major flavonol (rutin) in asparagus. Both compounds have four hydroxyl groups and a sugar molecule attached at the C-3 position. Unlike cyanidin, rutin has a carbonyl group at C-4 and a double bond between C-2 and C-3 positions. Rice-Evans et al. (1996) reported that the double bond between C-2 and C-3, in conjugation with the 4-oxo group, enhances the antioxidant activity. Cyanidin also contains a positive charge (flavylium cation). Even though the hydrogen donor capacities of polyphenols are proportional to the number of hydroxyl groups, it has been reported that DPPH radical activity is not consistent with the number of OH-moieties. Stratil et al. (2006) found that the DPPH scavenging activity of ferulic acid (one OH group) and caffeic acid (two OH groups) were equal and gallic acid (three OH groups) had five times higher values. Cheel et al (2007) also reported that total flavonoid content of strawberries had a better correlation with DPPH radical scavenging activity than total anthocyanins.

Total anthocyanin contents showed a significant difference among harvest dates but there was not a specific trend (**Figure 2,** $p \leq 0.05$). Anthocyanin content of asparagus varieties decreased from the first (April 25) to the second harvest date (May 17) and increased at the third harvest date (June 1).

Francis (1967) isolated four anthocyanin pigments from asparagus spears. The major pigment was a linear triglycoside, cyanidin-3-rhamnosylglucosylglucoside, followed by cyanidin-3-rhamnosylglucoside. The peonidin analogues of the above pigments were also detected in minor quantities. Sakaguchi et al. (2005) investigated the anthocyanin variation in asparagus species and worked on identification of anthocyanins present in asparagus. Eighteen green and four purple asparagus cultivars were analyzed for their anthocyanins and no variation of anthocyanin constitution was found within species and subspecies. They also could not find any relationship between sex expression of asparagus and anthocyanin constitution. Sakaguchi et al. (2005) reported that asparagus species have three anthocyanin compounds composing of cyanidin, glucose and either rhamnose or glucose.

Anthocyanin pigments are also present in white asparagus spears, which results in the development of purple color in the tips. White spears having purple coloration are considered to have inferior quality. Siomos et al. (1995) pointed out that anthocyanin synthesis in asparagus requires light exposure and anthocyanin accumulation continues in harvested spears even at a low storage temperature. Flores et al. (2005) studied the effect of phenylalanine ammonia-lyase (PAL) on the light-induced accumulation of anthocyanins in the asparagus tissue. Results indicated that light induced PAL activity, which in turn stimulated anthocyanin accumulation. Siomos et al. (2001) managed to

prevent postharvest anthocyanin synthesis of white asparagus with a CO_2 concentration ≥5% for spears held in the dark or a CO_2 concentration ≥10% for spears held in the light.

Phenolic composition of asparagus

Extracts of nine asparagus varieties were analyzed by HPLC-DAD. Representative HPLC chromatograms of green and purple asparagus cultivars are illustrated in **Figure 3**. The HPLC pattern of phenolic compounds was similar in all green varieties. 'Guelph Millenium' was selected to represent all green varieties since it contained the greatest amount of phenolics. On the basis of the 'Guelph Millenium' chromatogram given in **Figure 3A**, three significant peaks were observed at 7.7, 12.0 and 24.2 minutes for green asparagus. The phenolic profiles of green and purple varieties were slightly different with an additional peak at 26.7 min observed in the 'Purple Passion' chromatogram **Figure 3B**.

Several standards were used to identify the major phenolics in the asparagus. Both retention time on HPLC and characteristic spectrum obtained by DAD for each standard was compared with those of asparagus samples. The retention times obtained for authentic phenolic standards are illustrated in **Table 3**. The first peak in the asparagus chromatogram eluted at 7.7 min and its DAD spectrum showed a maximum absorption at 240 and 324 nm (**Figure 4**). None of the standards tested eluted at the same time (7.7 min) therefore this peak could not be identified. However, peak 1 appeared to be a flavone or flavonol based on its DAD spectrum, since UV spectra of flavones and flavonols have a band II peak at around 240-280 nm and a band I peak at 300-380 nm (Merken and Beecher, 2000). The second peak was identified as chlorogenic acid since

its retention time and DAD spectrum were coincident with the authentic chlorogenic acid. The elution time of chlorogenic acid was 11.9 min, corresponding to peak 2 in all asparagus cultivars (**Table 3**). Diode array spectrum of both chlorogenic acid standard and asparagus cultivars showed maximum absorption at 240 and 324 nm (**Figure 5**).

The major phenolic found in all asparagus varieties was rutin (**Figure 3**). The retention time of authentic rutin standard was 24.1 min, corresponding to peak 3 in all asparagus varieties (**Table 3**). Diode array spectra of the rutin standard and peak 3 from asparagus cultivars demonstrated a maximum absorption at 254 and 356 nm (**Figure 6**). Previous reports stated that rutin is the most abundant phenolic in asparagus. For example, Makris and Rossiter (2001) observed that rutin was the predominant peak in HPLC chromatogram of asparagus. Similar results were found by Wang et al (2003) who measured the bioactive compounds in asparagus using a LC/MS method with selected ion monitoring and reported rutin as the major phenolic.

Quantification of both chlorogenic acid and rutin were made by drawing calibration curves using external standards. For both standards, the correlation coefficient exceeded 0.999 and reliable standard curves were established for HPLC. However, chlorogenic acid did not elute as a sharp, single peak under these chromatographic conditions. Two isomers of chlorogenic acid coeluted at fractions of the same minute (12.1 and 12.3 min). The same elution pattern was observed in the corresponding peak of asparagus chromatograms. Thus quantification of chlorogenic acid was done as a single peak by adding the areas under the peaks of the two isomers. The chlorogenic acid contents of nine asparagus varieties at three different harvest dates are illustrated in **Table 4** with the data ranked from greatest to smallest value. The results ranged from

0.60 mg chlorogenic acid/g DW (53.3 μg/g FW) for 'UC 157' to 0.44 mg chlorogenic acid/g DW (39.5 μg/g FW) for 'Purple Passion'. The chlorogenic acid concentration was significantly different ($p<0.05$) among varieties. 'UC 157', 'Jersey Knight' and 'Morehouse Select' demonstrated significantly higher results than 'Guelph Millenium' and 'Purple Passion'. It was observed that chlorogenic acid concentration significantly increased at each harvest date through the season. Results are in agreement with Sakakibara et al (2003) who detected chlorogenic acid in asparagus using HPLC-DAD. They reported that asparagus contains chlorogenic acid at a level of 34.37-85.03 μg/g fresh edible part.

The rutin contents of nine varieties of asparagus at three different harvest dates are listed from greatest to smallest in **Table 5**. The results showed that rutin content varies from 1.39 to 2.00 mg/g DW (123.2 to 178.2 μg/g FW) among the nine varieties. 'Guelph Millenium' was found to have the largest rutin content while 'Purple Passion' had the smallest. Varietal differences in the rutin content measured by HPLC showed a similar trend compared to the results of colorimetric $AlCl_3$ method. No significant difference was found between all-male varieties including 'Guelph Millenium', Jersey varieties, 'Syn 4' and 'Morehouse Select'. Resembling the $AlCl_3$ data, 'UC 157' and 'Purple Passion' contained significantly smaller amounts of rutin (1.39 mg/g DW for both varieties). Linear regression was used to compare rutin concentrations obtained by HPLC analysis with the values derived from colorimetric ($AlCl_3$) method. A high correlation coefficient was found between the rutin concentrations obtained by the two methods ($R=0.93$, $p\leq0.05$). **Figure 7** shows a scatter graph of the data with best line of fit. Rutin content had previously been reported for Washington asparagus by Mulenburg

(1992) using HPLC and colorimetric $AlCl_3$ methods. The results of the current study showed values higher than that of Mulenberg (1992) since his study was performed on canned asparagus brine (113.2-148.1 µg/g FW). Mulenberg (1992) reported a good correlation between HPLC and the colorimetric $AlCl_3$ method (R=0.96). He found an average ratio of 1.08 between the rutin concentrations as measured by colorimetric/HPLC. In the present study, although a good correlation was found between the two methods, the rutin content obtained by HPLC was lower than that of colorimetric method. The difference can be due to different extraction methods used for each method. In the HPLC technique, a multi-step chemical purification was applied, while the colorimetric assay was performed on water extracts. It has also been reported that $AlCl_3$ is a less selective method than HPLC since $AlCl_3$ can react with other flavonoids in the samples. Kreft et al. (2002) found 30% higher results with $AlCl_3$ than HPLC.

Other studies found in the literature investigated rutin content of asparagus with HPLC. Wang et al. (2003) quantified rutin in the shoots of various asparagus breeding lines using a HPLC/MS system under both the positive and negative ion modes with UV detection at 255 nm. They found levels at 300-600 µg/g FW. Maeda and Kakuta (2005) used a HPLC system to quantify rutin in different colors of asparagus. They reported an average rutin value of 152.5 µg/g FW for green and 236 µg/g FW for purple asparagus cultivars. They could not detect any rutin in white asparagus spears. Makris and Rossiter (2001) reported that methanolic extracts of asparagus contained rutin at a level of 280.3 µg/g FW and that chopping and maceration caused a decrease as high as 18.5%. The decrease was attributed to the oxidation of rutin rather than hydrolysis since quercetin was not detected either in control or chopped asparagus. Similar to the study of Makris

and Rossiter (2001), no quercetin was found in any of the asparagus varieties in the present study. As can be seen from **Figure 3**, quercetin standard eluted at 30.7 min, where no similar peak was observed in asparagus chromatograms (**Table 3**).

Previous studies have presented the rutin concentration of asparagus somewhat higher than that shown in the current study. One possible reason for the discrepancy could be the differences in the extraction methods. Previous studies used a one-step extraction with either 70% ethanol or 80% methanol (Wang et al., 2003; Makris and Rossiter, 2001, respectively) while in the present study a multi-step purification was performed. To check the performance of the extraction method, 22 µg/mL rutin and chlorogenic acid were spiked into acidified extraction solvent of asparagus and at each step of the extraction, recovery of the standards were analyzed. Recoveries of rutin and chlorogenic acid in the final extract were found as 54.9 and 59.4%, respectively. These results indicated that some of the phenolic compounds were lost during the extraction. Pretreatment of asparagus samples with an acidic extraction solution might cause losses due to the decomposition and polymerization of polyphenols. For example, Hertog et al (1992) reported that under optimal conditions, hydrolysis led to an underestimation of up to 50% of the true polyphenol level in food. Additionally, the results might be affected by the length and temperature of evaporation used for the removal of extraction solvents. Several filtration and separation steps might be other factors which cause losses. Sakakibara et al. (2003) developed a method for simultaneous quantification of all polyphenols in fruits, vegetables and tea using HPLC-DAD. In this study, 70% and 77% recovery were reported for chlorogenic acid and rutin, respectively. The possible reasons for the loss of polyphenols were explained as the pretreatment steps involving

homogenization in liquid nitrogen, lyophilization and extraction with 90% methanol. When it is considered that a multi-step purification was applied in the present study the lower recovery levels are understandable. In addition, the coefficient of variation of the repeatability and reproducibility was good, being less than 1.13% and 0.98%, respectively. The repeatability and reproducibility of the present study were similar or better than those of other studies (Hakkinen et al., 1998; Sakakibara et al., 2003).

HPLC-DAD profiles of 'Purple Passion' revealed the presence of a unique phenolic compound. Different than the HPLC chromatograms of green asparagus varieties, a peak eluted at 26.7 min in the purple asparagus ('Purple Passion') chromatogram (**Figure 3**). The detected phenolic peak was first compared with respect to retention time with those of reference standards. The possible four reference standards were phloridzin, myricetin, fisetin and eriodictyol which eluted at 25.7, 25.9, 27.3 and 27.5 minutes, respectively (**Table 3**). The spectrum of peak 4 in purple asparagus was compared with that of the four standard compounds. As can be seen from **Figure 8**, the DAD spectrum of purple asparagus showed maximum absorption only at 246 nm. The DAD spectra of phloridzin, myricetin, fisetin and eriodictyol are illustrated in **Figure 9**. Each of these standards exhibited different absorbance patterns than peak 4. Therefore, the unique peak in the HPLC chromatogram of purple asparagus was not defined. Merken and Beecher (2000) reported that flavonoid compounds have a bimodal absorption pattern with the first absorbance maximum in the UV-B or UV-C range (240-280 nm) and the second above 300 nm. Since the DAD spectrum of peak 4 showed maximum absorption at only one wavelength, it was concluded that the unknown compound is not a flavonoid.

Several small peaks were observed after 47 min both in green and purple asparagus chromatograms. Those peaks also appeared in methanol (blank) chromatogram which confirms that they are artifacts resulting from the gradient system itself.

There are some other studies in the literature reporting the presence of some minor phenolics in asparagus. Sakakibara et al (2003) reported that in addition to quercetin glycosides (rutin), asparagus contains caffeic acid (2.34-10.27 μg/g FW), chlorogenic acid (34.37-85.03 μg/g FW) and cinnamic acid (2.52-23.71 μg/g FW). Makris and Rossiter (2001) detected five minor phenolics (quercetin and kaempferol glycosides) other than rutin in asparagus spears. Wang et al (2003) detected protodiosicin in asparagus using LC/MS under positive and negative ion modes. Ferulic and p-coumaric acids have been detected in the cell wall material of asparagus and are predominantly concentrated in the basal region of the spear (Rodriguez-Arcos et al., 2002; 2004; 2005). Some of these phenolics were not detected in the present study because those compounds are found at very low levels in asparagus. These minor phenolics could be lost during the extraction process.

HPLC-DPPH analysis of antiradical compounds

A new screening technique for antioxidants was used by HPLC using the DPPH free radical. The spectrophotometric DPPH radical scavenging method is a widely used assay to measure antioxidant activity. Upon the reaction of radical with antioxidants, the DPPH radical is reduced by gaining a hydrogen atom from the antioxidant and forms the stable DPPH-H molecule. As a result of this reduction reaction, the color of the radical changes from purple to yellow which leads to a decrease in the absorbance. A similar

mechanism was used with the HPLC-DPPH method. Before starting the analysis of extracts it was essential to determine the reaction time to be used in the assay. For this purpose, the reaction kinetics of potential standards with 5 mM DPPH radical were measured spectrophotometrically using the DPPH free radical scavenging method (Brand-Williams et al., 1995). The reaction kinetics of chlorogenic acid, rutin, quercetin and quercetin-3-glucoside are illustrated in **Figure 10**. As it is shown in the figure, quercetin reacted most rapidly with the DPPH radical and reached the plateau at approximately 5 min. It was followed by rutin and quercetin-3-glucoside which came to the end of the reaction at around 30 minutes. The reaction of chlorogenic acid was significantly slower than other phenolics tested, reaching a steady state after 60 min. Based on the kinetic behaviors of phenolic standards, the reaction time with the DPPH radical before the HPLC analysis was fixed at 60 min.

An aliquot of asparagus extract was allowed to react with 5 mM DPPH radical for 60 min. Phenolic compounds having strong antioxidant activity were consumed by the reaction with added DPPH radical. The extract/radical mixture was then measured by HPLC. As a control, the same asparagus extract was analyzed using HPLC without adding the DPPH. Peaks corresponding to the strong antioxidants disappeared or changed to other peaks in the asparagus chromatogram after the reaction. The antioxidative compounds were detected as the corresponding decreased or lost peak on the HPLC chromatograms. **Figure 11** illustrates the chromatograms of green asparagus extract (‘Guelph Millenium’) before and after the addition of the DPPH radical. The peak corresponding to rutin (24.1 min) in **Figure 11A** disappeared almost completely by the addition of the DPPH and another peak appeared at 20.5 min (**Figure 11B)**. The peak

loss indicated that rutin is possibly the most active antiradical compound in the green asparagus extract. The pure rutin standard was analyzed by HPLC under the same conditions as described above with and without the addition of the DPPH radical. After the reaction with the DPPH radical, the peak at 24.1 min disappeared and a new peak emerged at 20.5 min. The occurrence of the new peak suggested the formation of an oxidized product of rutin after the reaction with the DPPH. Antioxidative compounds donate a hydrogen atom to the radical and become oxidized. The polarity of oxidized flavonoids is significantly higher than that of the parent compounds allowing them to elute more rapidly from the C_{18} column (Jungbluth and Ternes, 2000). As can be seen in **Figure 11**, the two minor peaks eluting at 7.7 min and 12.0 min (chlorogenic acid) (**Figure 11A**) disappeared and a new peak was formed at 6.2 min (**Figure 11B**). Therefore, chlorogenic acid and peak 1 were identified as the other antiradical compounds in the green asparagus extract. The pure chlorogenic acid standard was analyzed under the same conditions with and without the addition of the DPPH radical. After the reaction with the DPPH radical, the peak eluting at 12.0 min disappeared and a new peak emerged at 6.2 min. The new peak in the second chromatogram was referred to the oxidized products of chlorogenic acid. However, the molar concentration of the new peak formed by the reaction of pure chlorogenic acid was smaller than that formed by asparagus extracts. It is suggested that the oxidized products of peak 1 also contributed to the formation of the new peak. Therefore, it appeared that chlorogenic acid and peak 1 have similar oxidized products. When the DAD spectra of chlorogenic acid and peak 1 are compared (**Figure 4 and 5)**, it is observed that both compounds exhibited the same

absorbance pattern. These results suggested that peak 1 may have a similar structure to chlorogenic acid, such as an isomer or another cinnamate derivative.

The analytical data for the extract of purple asparagus variety ('Purple Passion') are shown in **Figure 12**. The similar pattern for the disappearance of rutin and chlorogenic acid in green asparagus was observed for purple variety. As is shown in **Figure 12B,** the intensity of the peak corresponding to rutin at 24.0 min decreased markedly after the reaction with the DPPH and a new peak corresponding to oxidized rutin was formed at 20.5 min. The peak intensities of chlorogenic acid (12.0 min) and the unknown compound (7.7 min) in purple asparagus were also distinctly decreased (**Figure 12B**) when compared with these in the first chromatogram (**Figure 12A**). Oxidized products of chlorogenic acid and peak 1 formed at 6.2 min. It is noteworthy to say that there was no change in the intensity of the unique peak eluting at 26.6 min in the purple asparagus chromatogram. This data indicated that the unique compound (peak 4) in the purple asparagus variety did not possess antiradical activity. Bandoniene et al. (2002) used an online HPLC-DPPH method with a post column reaction to measure the antioxidant activity of several phenolic standards such as gallic acid, chlorogenic acid, catechin, epicatechin, rutin, phloridzin and quercetin. It was observed that phloridzin was the only compound which did not possess any antiradical activity. In the present study, the retention time and DAD spectrum of phloridzin standard was compared to the peak 4 of the purple asparagus chromatogram. Although the retention times of peak 4 and phloridzin were similar, DAD spectra showed that phloridzin did not correspond to the peak 4 of the purple asparagus chromatogram.

DPPH radical itself was also analyzed using HPLC under the same conditions. The HPLC data of the DPPH revealed that the large peak eluted at 48.2 min in both **Figure 11B** and **12B** corresponds to the DPPH radical remaining after the reaction with extracts. A smaller peak was also observed at 47.3 min in both **Figure 11B** and **12B.** The appearance of this peak may due to the formation of stable 1,1-diphenyl-2-picrylhydradine (DPPH-H), a hydrogen absorbed form of the DPPH, after reduction reaction (Masuda et al., 2003).

The intensity of the peaks eluting after 47 min did not change with the reaction of the DPPH radical. It was confirmed that those peaks do not correspond to flavonoids and they do not have antioxidant activity (**Figure 11** and **12**). They are artifacts caused by the gradient system.

HPLC-DPPH data suggest that rutin, chlorogenic acid and the unknown compound (peak 1) are the main antiradical compounds in both green and purple asparagus varieties. However, some studies claim that the efficiency of antioxidants depends on how fast they can react with the radical compound (Sanchez-Moreno et al., 1998). It has been suggested that the more rapidly the absorbance decreases, the more potent the antiradical activity of the compound in terms of hydrogen donating ability (Gadow et al., 1997). For this reason, the efficiency of antiradical compounds in asparagus extracts was tested by measuring their reaction kinetic with the DPPH radical. Both green and purple asparagus extracts were mixed with the DPPH radical and the decrease in absorbance was measured using the HPLC-DPPH method 0, 15, 30, 60 and 90 minutes after the addition of the DPPH radical. The efficiency of antiradicals was determined by the remaining area under the peaks after each duration of reaction (**Table**

6). The percent decrease in the amount of antioxidants was calculated and plotted against the time of reaction. **Figure 13** illustrates the reaction kinetics of antiradical compounds in green and purple asparagus varieties. In both types of asparagus the unknown compound (peak 1) was consumed completely by the DPPH radical before 15 min. Rutin showed a strong antiradical activity. The reaction reached a plateau at 30 minutes and approximately 93% of rutin in asparagus was consumed by the DPPH radical. Chlorogenic acid exhibited a slower reaction than rutin and reached a steady state at 60 min and 75% decrease was observed from the original amount of chlorogenic acid in asparagus extracts. The difference in reaction rate of peak 1 and chlorogenic acid led to a question about the similarity of their structures. These two compounds were found to have similar absorbance spectra and their DPPH oxidation products eluted at the same time. Therefore it was concluded that these compounds may have similar structures such as isomers. However, peak 1 reacted with the DPPH radical more rapidly than chlorogenic acid. The number of caffeoyl groups in peak 1 could be different in addition to their positions. Iwai et al (2004) reported that the antioxidative activity of hydroxycinnamic acid derivatives depends on the number of caffeoyl groups rather than the linkage positions of caffeoyl groups on the quinic core. However, Iwai et al (2004) did not report kinetic data of chlorogenic acid isomers.

As is seen in **Figure 13B,** the unknown compound in purple asparagus remained the same and did not react with the DPPH radical. It is shown that this compound does not possess hydrogen donating ability. Although rutin was found to be one of the strongest and fastest antiradical compound in asparagus, in a study comparing the antiradical efficiency of polyphenols, the reaction kinetic of rutin was classified as slow

compared to other phenolics such as gallic acid, tannic and caffeic acid (Sanchez-Moreno et al., 1998). Bandoiene et al (2002) analyzed several phenolic compounds using online HPLC-DPPH system and reported that rutin and chlorogenic acid were among the phenolics showing slow reaction with the DPPH. However, their results supported that rutin has a stronger and faster activity than chlorogenic acid. It is also arguable to claim that phenolics showing more rapid reaction have greater antioxidant activity. Because slow-reacting compounds maintain their activity for longer time and provide protection against free radicals for an extended period.

CONCLUSION

Phenolic constituents and anthocyanin content of nine asparagus varieties were measured after a multi-step fractionation. The anthocyanin content measured spectrophotometrically varied between varieties and 'Purple Passion' showed around 4 times greater amounts than green cultivars. The phenolic profiles of nine asparagus varieties were determined using a HPLC-DAD system. Rutin and chlorogenic acid were found to be the major phenolics in all varieties tested. 'Purple Passion' exhibited a unique peak which could not be identified. The concentration of phenolic compounds varied from one cultivar to another and in relation to harvest date. Rutin was the predominant phenolic compound and its level ranged from 2.00 to 1.39 mg/g DW. A high correlation coefficient was found between the rutin concentrations obtained by HPLC and the colorimetric $AlCl_3$ methods.

HPLC coupled to a DAD and a DPPH-based antioxidant assay provided a powerful technique for the rapid screening of antioxidant compounds in asparagus

extracts. The technique was simple and easily applicable to the measurement and comparison of antiradical efficiency of phenolic compounds in asparagus. The antioxidative activity of each phenolic was measured by the decrease of the peak area after the precolumn reaction with the DPPH radical. Rutin and chlorogenic acid were identified as antiradical compounds in both green and purple asparagus varieties. The present study was first to find that rutin is the main antioxidant in asparagus.

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Table 1. Statistical analysis for total anthocyanins, rutin and chlorogenic acid contents

	Total Anthocyanins		Rutin		Chlorogenic acid	
Source of variation	F value	*p* value	F value	*p* value	F value	*p* value
Variety	38.79	< 0.0001*	6.15	< 0.0001*	7.28	< 0.0001*
Harvest date	32.96	< 0.0001*	20.92	< 0.0001*	52.66	< 0.0001*
Sampling plot	1.76	0.1825	2.25	0.1166	8.69	0.0006*
Variety*harvest date (interaction)	1.90	0.0442*	1.88	0.0460*	1.67	0.0852

***** *p* values ≤ 0.05 show statistically significant difference.

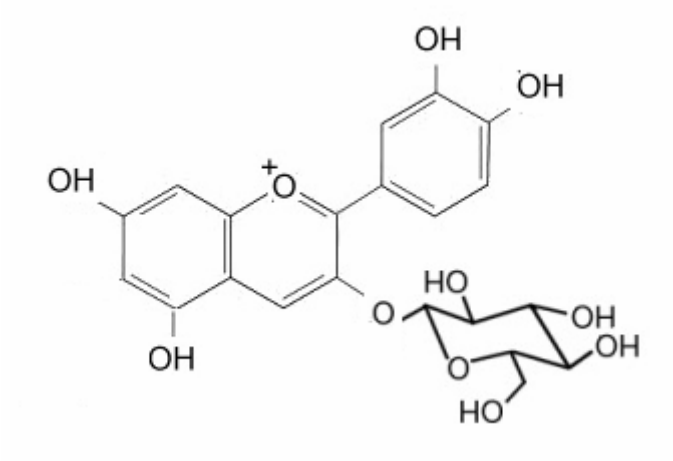
Total anthocyanins were determined spectrophotometrically. Rutin and chlorogenic acid contents were measured by HPLC analysis.

Table 2. Anthocyanin contents of nine asparagus varieties at three different harvest dates

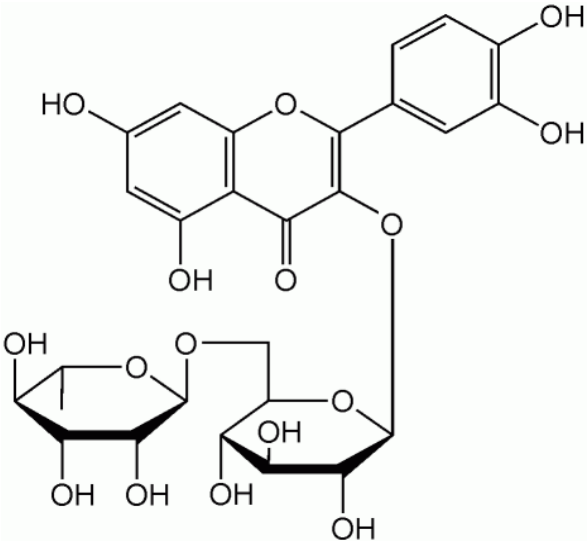
Variety	1st harvest	2nd harvest	3rd harvest	Mean ± std[1]
Purple Passion	1.27 ± 0.17	0.99 ± 0.15	0.99 ± 0.28	**1.08 ± 0.16[a]**
Jersey Giant	0.48 ± 0.06	0.28 ± 0.06	0.49 ± 0.02	**0.42 ± 0.12[b]**
Jersey Supreme	0.50 ± 0.04	0.26 ± 0.09	0.47 ± 0.05	**0.41 ± 0.13[b]**
Jersey Deluxe	0.47 ± 0.03	0.16 ± 0.15	0.48 ± 0.01	**0.37 ± 0.18[b]**
Syn 4	0.37 ± 0.07	0.37 ± 0.03	0.41 ± 0.04	**0.38 ± 0.02[bc]**
Jersey Knight	0.46 ± 0.06	0.24 ± 0.02	0.39 ± 0.01	**0.36 ± 0.11[bc]**
Morehouse Select	0.37 ± 0.02	0.12 ± 0.10	0.28 ± 0.02	**0.26 ± 0.13[cd]**
Guelph Millenium	0.39 ± 0.04	0.17 ± 0.04	0.23 ± 0.01	**0.26 ± 0.11[cd]**
UC 157	0.24 ± 0.05	0.17 ± 0.01	0.23 ± 0.05	**0.21 ± 0.04[d]**

Values are expressed as mg total anthocyanins/g dry weight asparagus and represent means ± SD (n=3)

[1]Means with different letters indicate significant differences ($P \leq 0.05$) by Tukey's test



(A) Cyanidin-3-glucoside



(B) Rutin

Figure 1. Chemical structure of (A) cyanidin-3-glucoside and (B) rutin

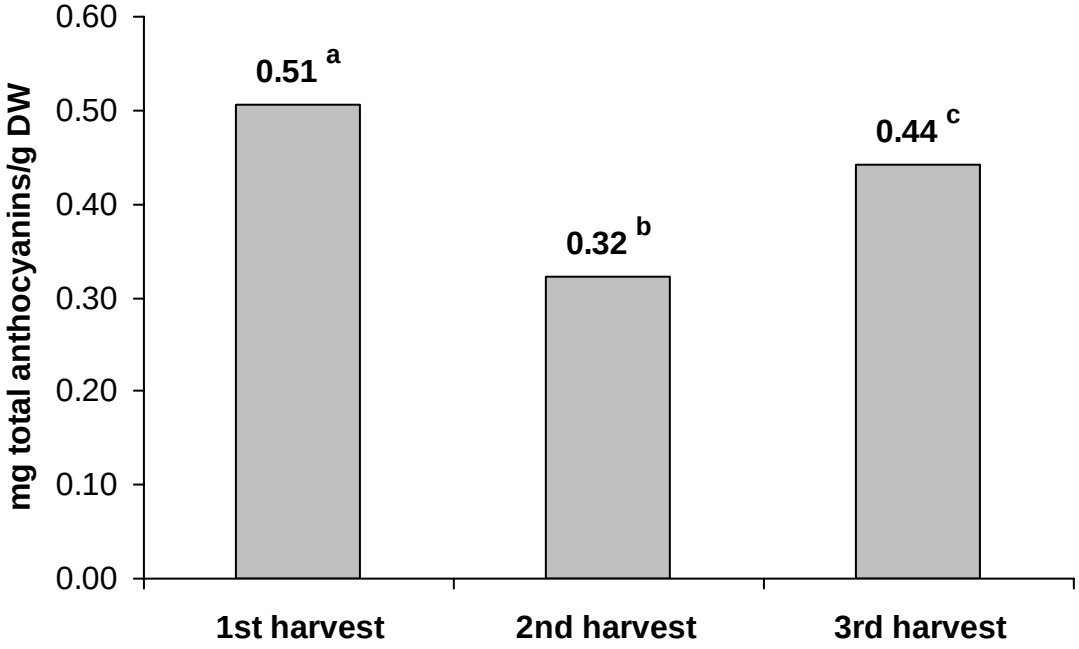


Figure 2. Total anthocyanin content of asparagus at different harvest dates

* The data are means of nine varieties of asparagus

* Means with different letters indicate significant differences ($P \leq 0.05$) by Tukey's test

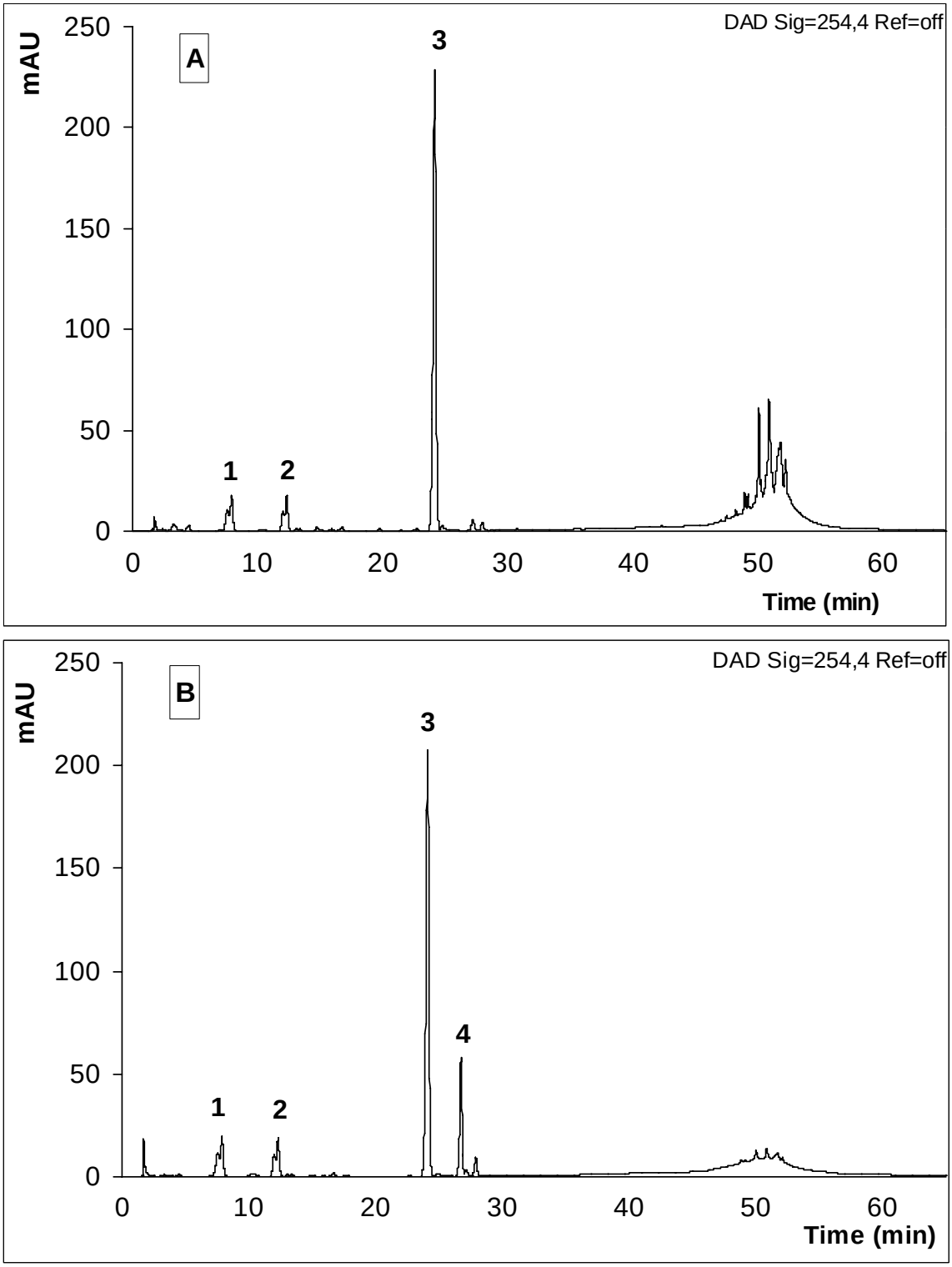


Figure 3. HPLC chromatograms of (A) green asparagus ('Guelph Millenium'), (B) purple asparagus ('Purple Passion') at λ=254 nm

Table 3. Retention times of phenolic standards

Phenolic compound	Elution time (min)
gallocatechin	5.2
catechin	8.9
chlorogenic acid	11.9
caffeic acid	13.0
epicatechin	14.4
gallocatechin gallate	15.3
ferulic acid	18.2
rutin	24.1
gossypetin	24.6
ellagic acid	24.7
phlorodzin	25.7
myricetin	25.9
fisetin	27.3
eriodictyol	27.5
cinnamic acid	29.1
daidzein	29.2
taxifolin	30.7
quercetin	30.7
naringenin	31.4
homoeriodictyol	31.7
phloretin	32.4
luteloin	32.5
hesperetin	33.0
coumarin	33.2
kaempferol	34.7
pinosylvin	35.6
isosakuranetin	39.8
apigenin	42.6
flavanone	43.6

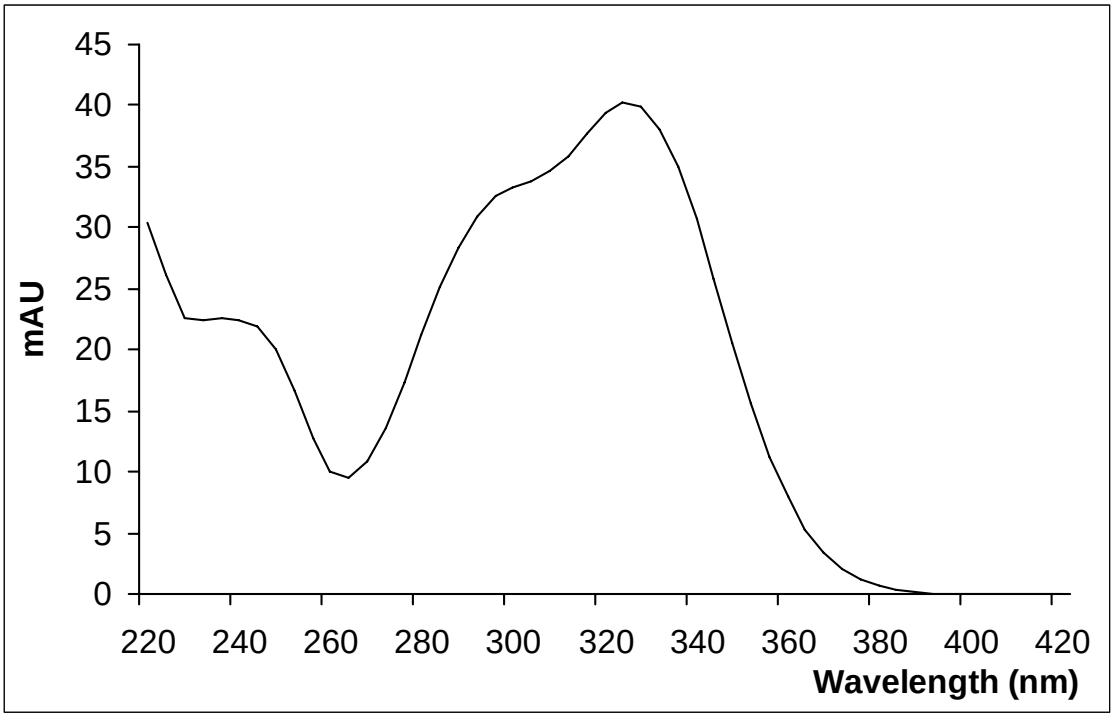


Figure 4. Diode array spectrum of the first peak of both green and purple asparagus

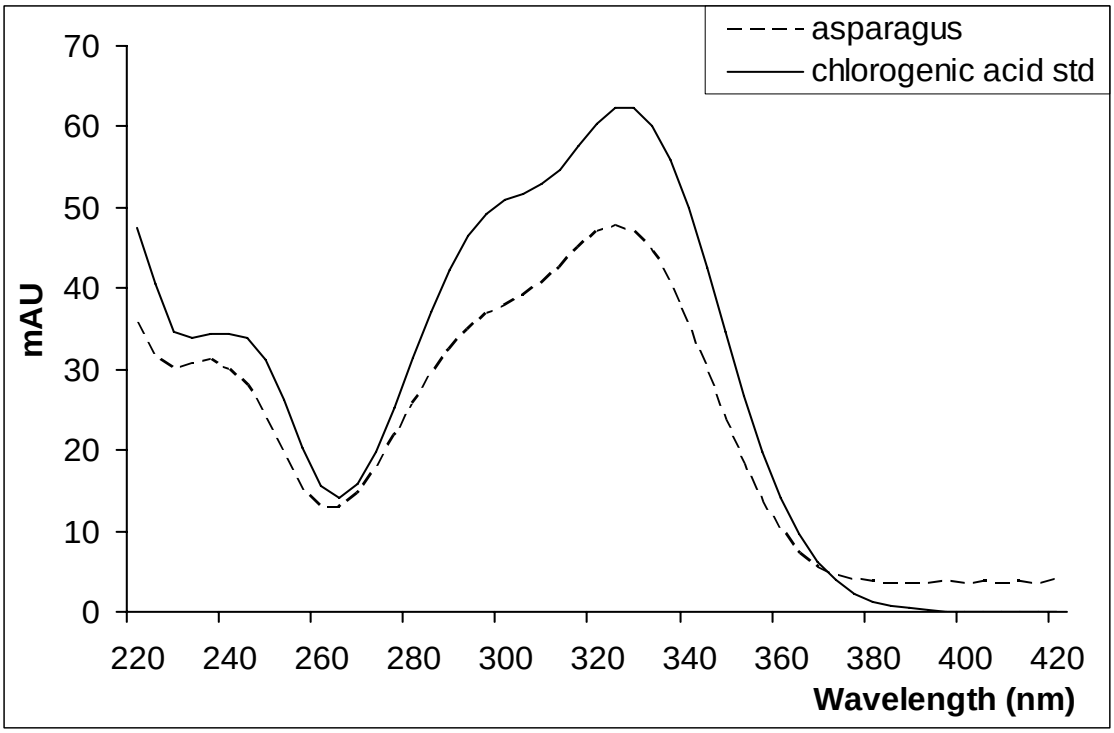


Figure 5. Diode array spectra of chlorogenic acid standard and the second peak of asparagus varieties.

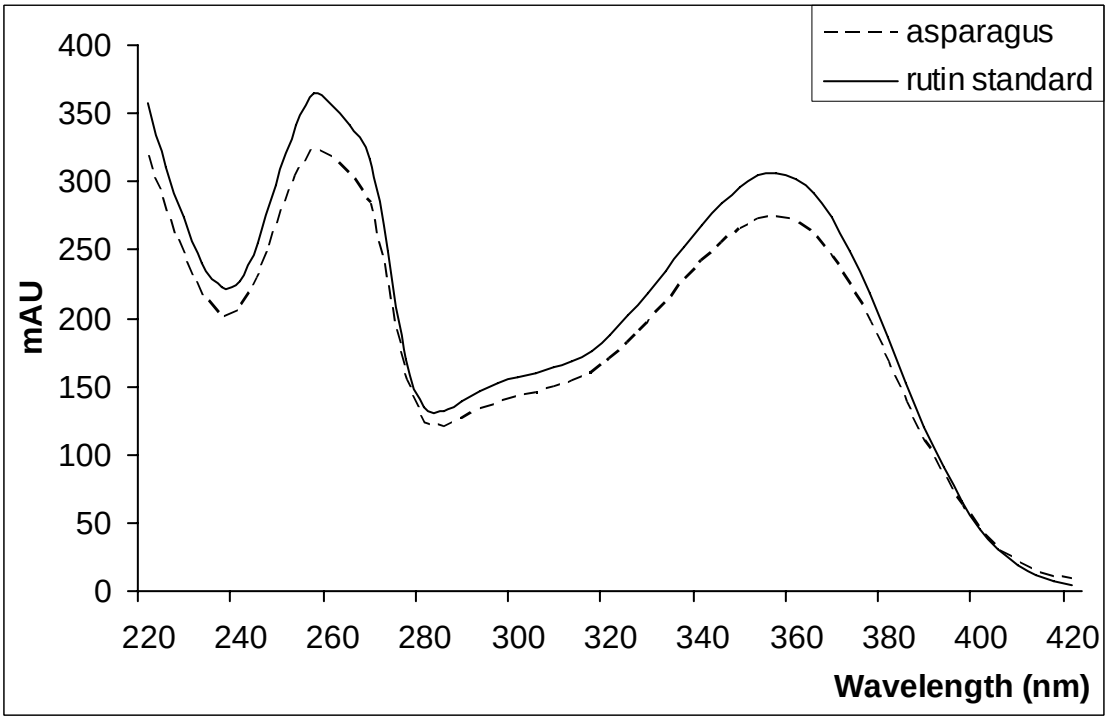


Figure 6. Diode array spectra of rutin standard and the third peak of asparagus varieties

Table 4. Chlorogenic acid contents of nine asparagus varieties at three different harvest dates measured by HPLC/DAD

Variety	1st harvest	2nd harvest	3rd harvest	Mean ± std[1]
UC 157	0.47 ± 0.05	0.55 ± 0.01	0.78 ± 0.04	**0.60 ± 0.16[a]**
Jersey Knight	0.45 ± 0.04	0.60 ± 0.06	0.71 ± 0.14	**0.59 ± 0.13[a]**
Morehouse Select	0.49 ± 0.09	0.66 ± 0.22	0.61 ± 0.06	**0.58 ± 0.09[a]**
Jersey Deluxe	0.42 ± 0.02	0.61 ± 0.05	0.59 ± 0.04	**0.54 ± 0.10[ab]**
Jersey Supreme	0.42 ± 0.01	0.53 ± 0.04	0.66 ± 0.09	**0.54 ± 0.13[ab]**
Jersey Giant	0.40 ± 0.02	0.59 ± 0.03	0.53 ± 0.13	**0.51 ± 0.10[ab]**
Syn 4	0.37 ± 0.05	0.46 ± 0.03	0.57 ± 0.04	**0.47 ± 0.10[ab]**
Guelph Millenium	0.38 ± 0.03	0.44 ± 0.01	0.51 ± 0.03	**0.45 ± 0.07[b]**
Purple Passion	0.36 ± 0.11	0.44 ± 0.10	0.53 ± 0.16	**0.44 ± 0.08[b]**

Values are expressed as mg chlorogenic acid/g dry weight asparagus and represent means ± SD (n=3)

[1]Means with different letters indicate significant differences ($P \leq 0.05$) by Tukey's test

Table 5. Rutin contents of nine asparagus varieties at three different harvest dates measured by HPLC/DAD

Variety	1st harvest	2nd harvest	3rd harvest	Mean ± std[1]
Guelph Millenium	1.74 ± 0.08	1.83 ± 0.18	2.43 ± 0.29	**2.00 ± 0.38[a]**
Syn 4	1.66 ± 0.35	1.63 ± 0.13	2.00 ± 0.13	**1.76 ± 0.21[a]**
Jersey Knight	1.76 ± 0.33	1.58 ± 0.13	1.92 ± 0.27	**1.76 ± 0.17[a]**
Jersey Giant	1.54 ± 0.14	1.74 ± 0.36	1.96 ± 0.28	**1.75 ± 0.21[a]**
Jersey Supreme	1.50 ± 0.10	1.50 ± 0.14	2.18 ± 0.17	**1.73 ± 0.39[ab]**
Morehouse Select	1.95 ± 0.34	1.48 ± 0.30	1.64 ± 0.03	**1.69 ± 0.24[ab]**
Jersey Deluxe	1.64 ± 0.08	1.56 ± 0.06	1.86 ± 0.10	**1.69 ± 0.16[ab]**
UC 157	1.38 ± 0.27	1.33 ± 0.08	1.46 ± 0.39	**1.39 ± 0.07[b]**
Purple Passion	1.22 ± 0.15	1.15 ± 0.28	1.79 ± 0.09	**1.39 ± 0.35[b]**

Values are expressed as mg rutin/g dry weight asparagus and represent means ± SD (n=3)

[1]Means with different letters indicate significant differences ($P \leq 0.05$) by Tukey's test

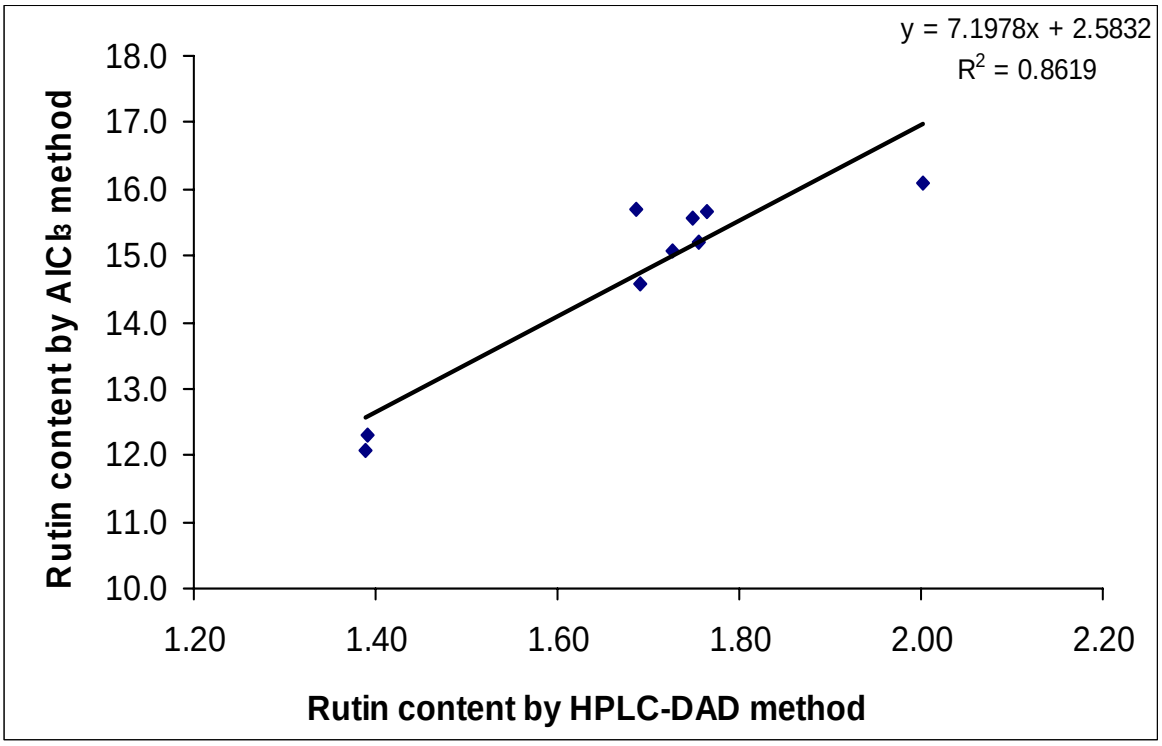


Figure 7. Correlation between HPLC and colorimetric $AlCl_3$ method for determination of rutin content (R=0.93)

* Values are expressed as mg rutin/g dry weight asparagus and represent means of three harvest dates for each variety (n=3).

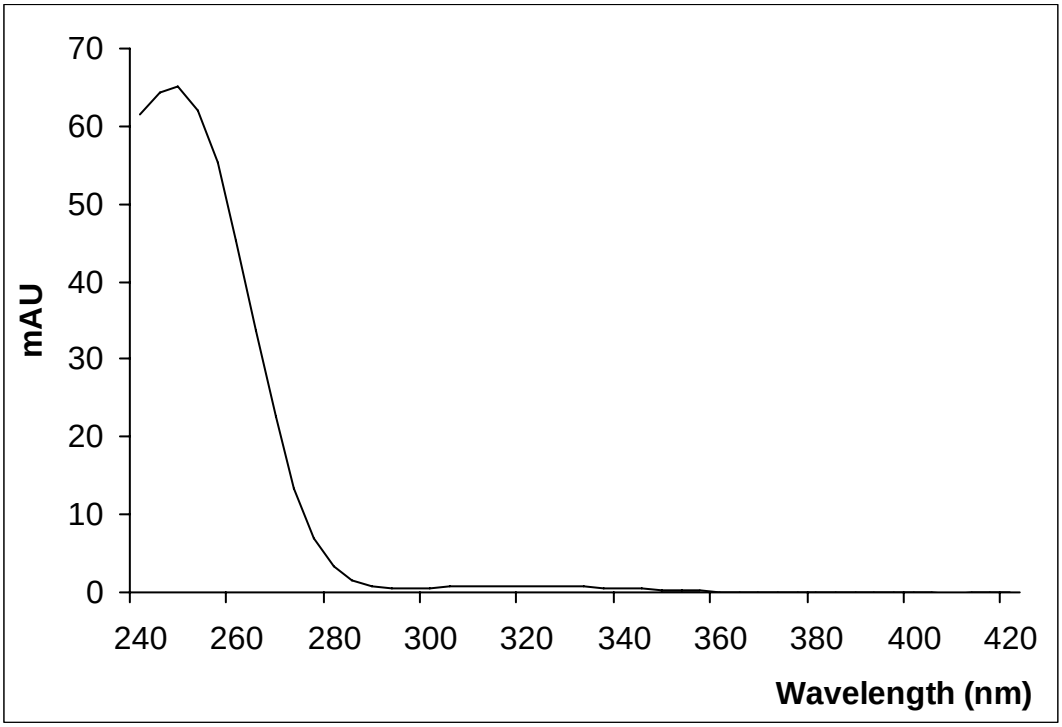


Figure 8. Diode array spectrum of the fourth peak of purple asparagus

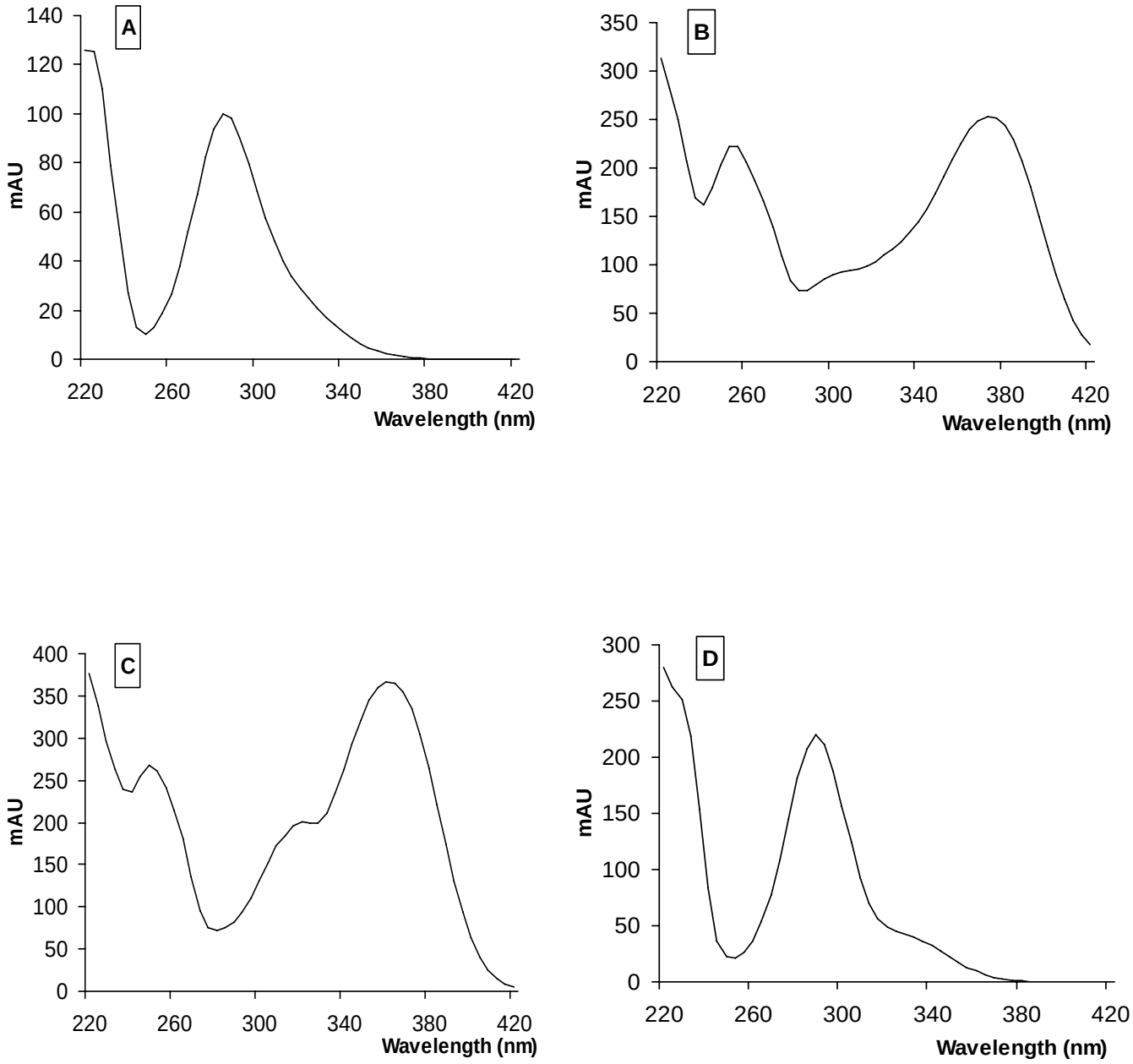


Figure 9. Diode array spectrum of (A) phloridzin, (B) myricetin, (C) fisetin and (D) eriodictyol

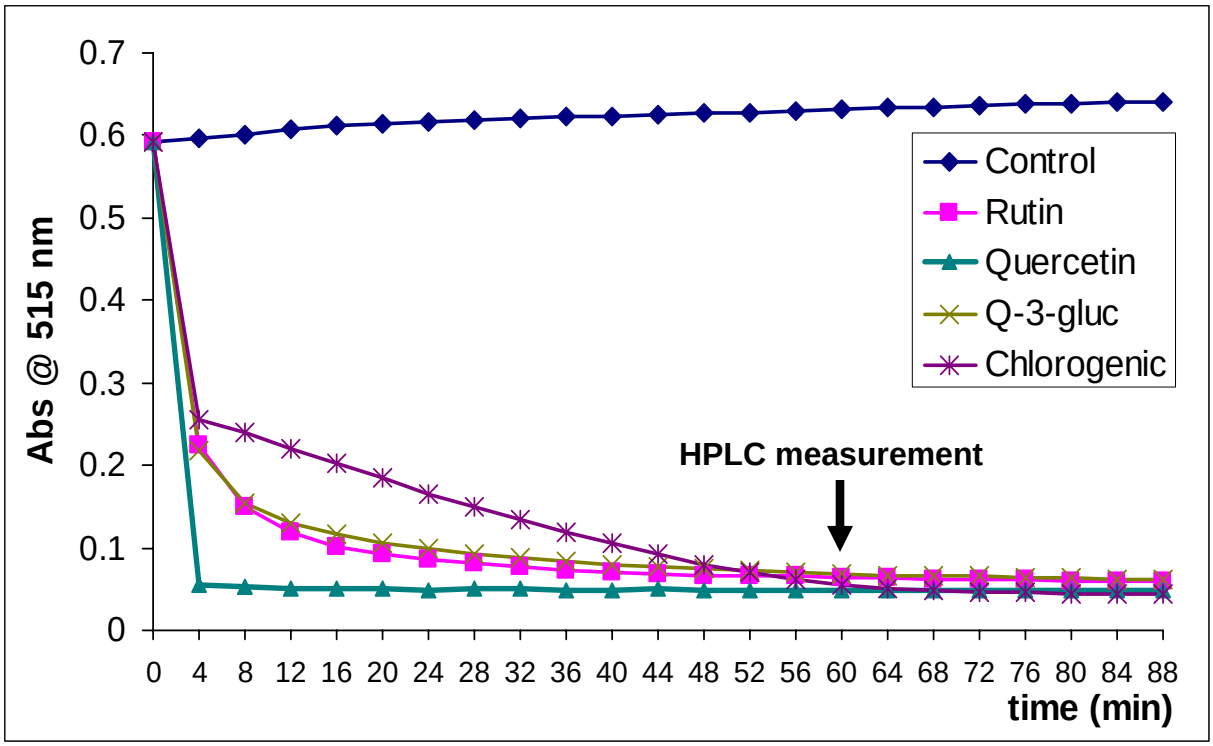


Figure 10. Reaction kinetics of phenolic standards with the DPPH radical during 90 min

* The final concentration of each standard was 2 mM.

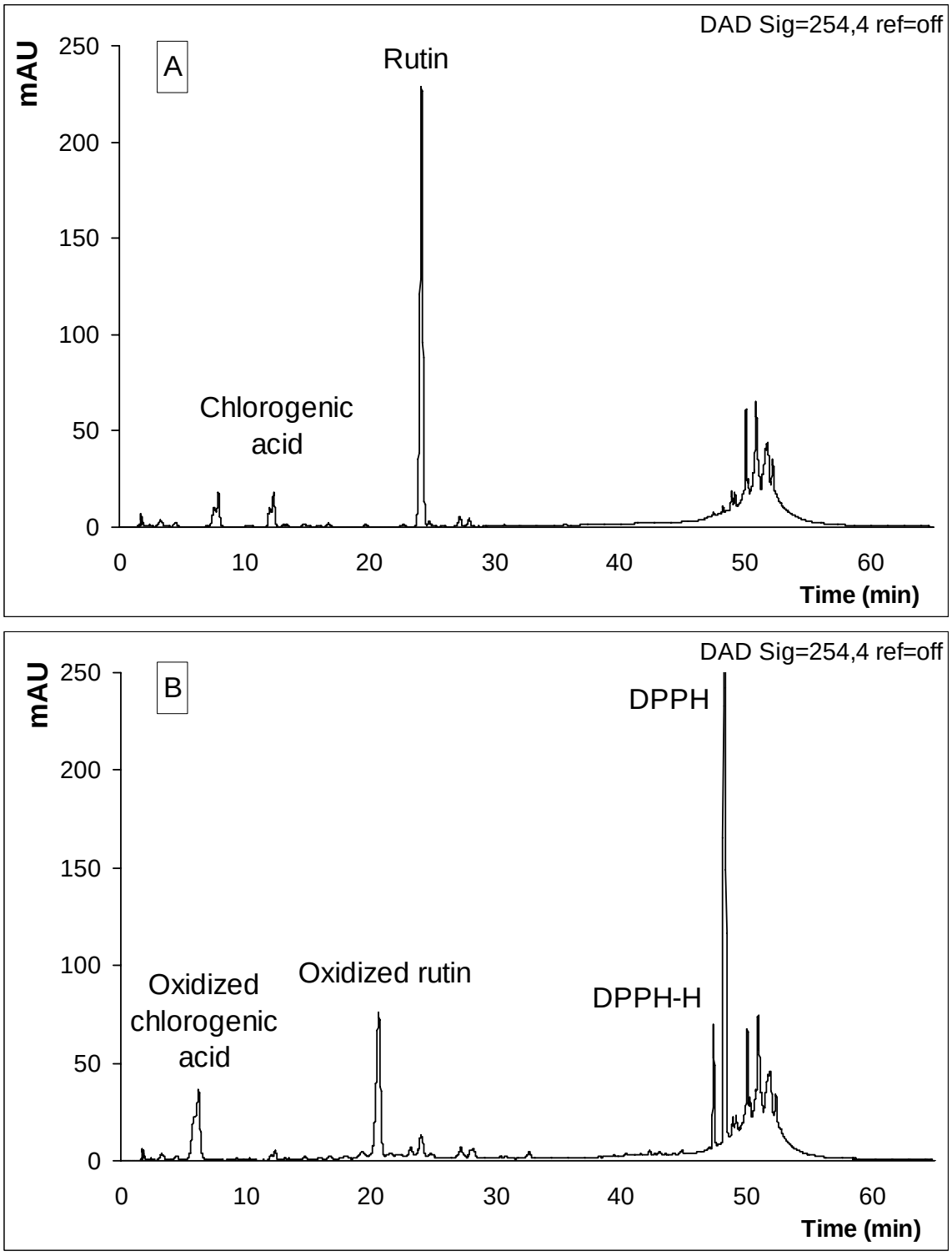


Figure 11. DPPH radical quenching chromatograms of green asparagus extract ('Guelph Millenium') (A) before and (B) 60 min after the addition of 5.0 mM DPPH radical

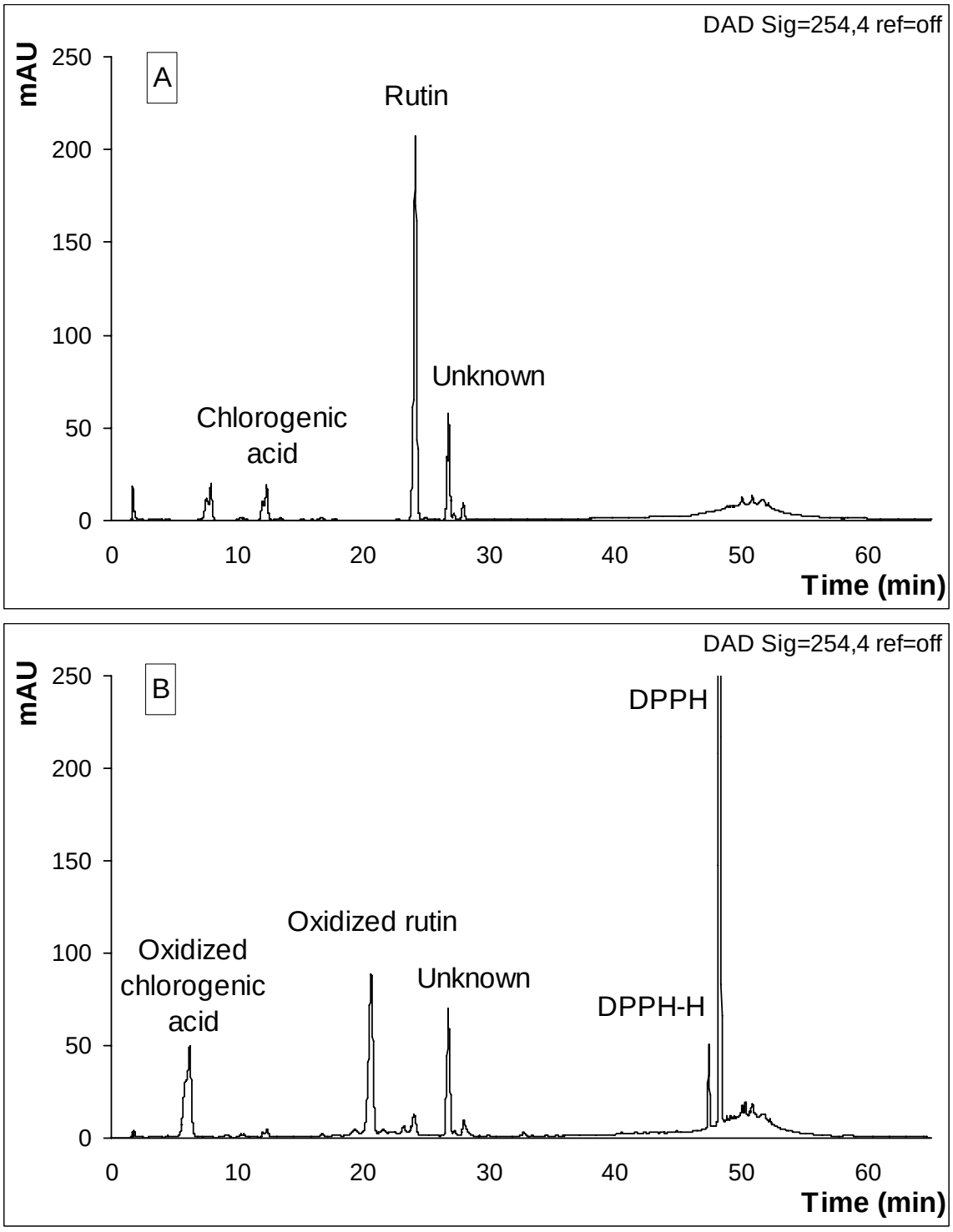


Figure 12. DPPH radical quenching chromatograms of purple asparagus extract ('Purple Passion') (A) before and (B) 60 min after the addition of 5.0 mM DPPH radical

Table 6. The area remaining under the peaks of phenolic compounds after certain times of the DPPH radical addition.

Time (min)	Green varieties			Purple varieties			
	Peak 1	Chlorogenic acid	Rutin	Peak 1	Chlorogenic acid	Rutin	Peak 4
0	431.99	381.98	3681.02	480.91	414.00	3300.62	1203.95
15	0	180.36	333.59	0	205.81	396.81	1207.24
30	0	127.73	260.71	0	146.85	290.57	1213.35
60	0	105.26	260.72	0	105.26	260.72	1216.35
90	0	104.87	260.70	0	104.75	259.56	1215.43

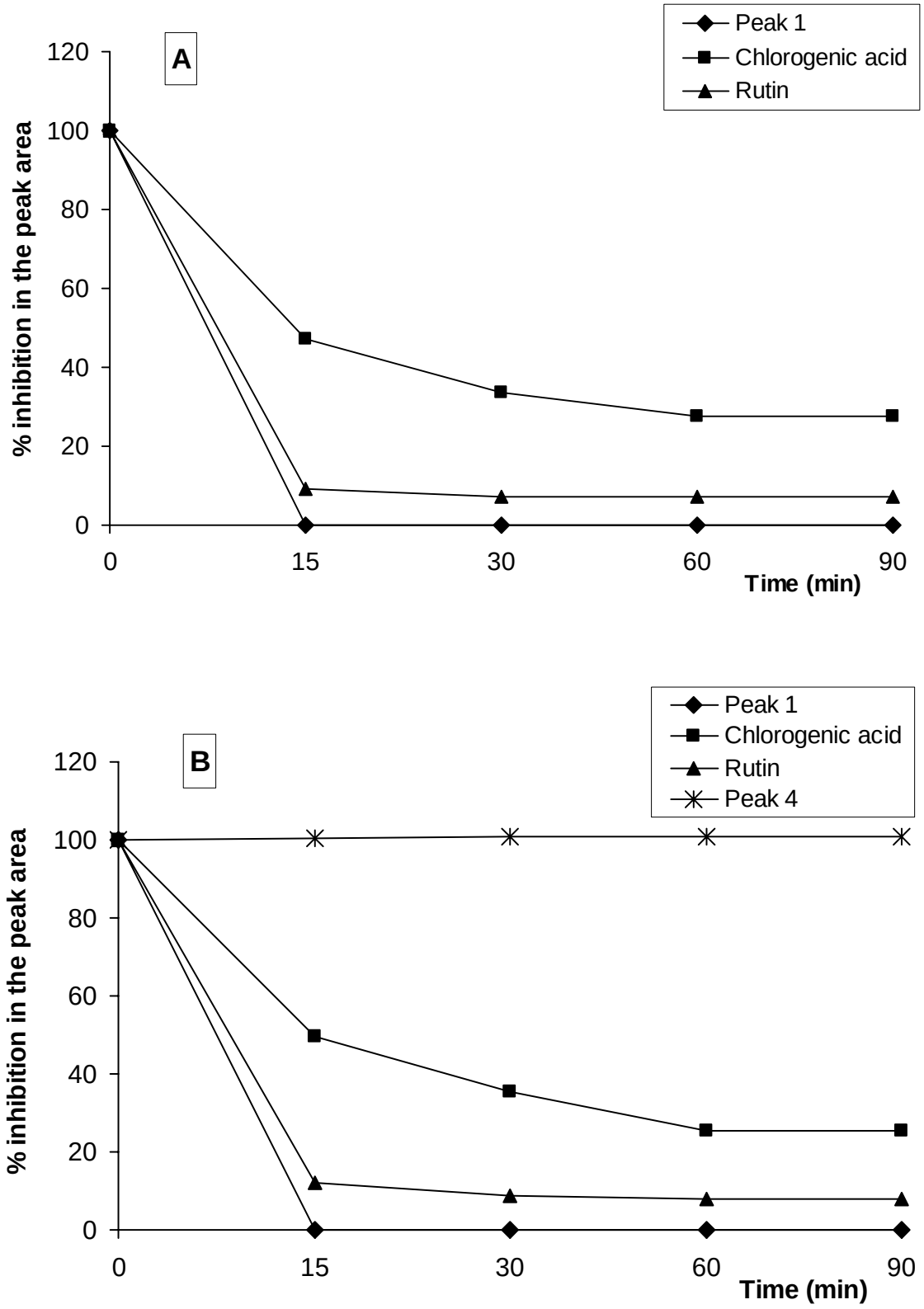


Figure 13. Reaction kinetics of (A) green and (B) purple asparagus measured by the HPLC-DPPH method after the addition of 5.0 mM DPPH radical

SUMMARY

Varietal and seasonal changes in total phenolics, rutin content, antioxidant activity and anthocyanin content of asparagus were investigated. Phenolic profiles of each variety were determined by a HPLC/DAD and antiradical compounds were identified using HPLC coupled with DPPH radical scavenging assay. This study revealed that the phenol content and associated antioxidant activity of cultivated asparagus changes due to genetic variation and harvest dates. All-male hybrid varieties, 'Guelph Millenium', 'Jersey Knight', 'Jersey Deluxe', 'Jersey Supreme', 'Jersey Giant', 'Morehouse Select', and 'Syn 4' showed significantly greater total phenolic and rutin contents as well as greater antioxidant activity than dioecious varieties, 'Purple Passion' and 'UC 157'. Evaluation of the harvest dates also resulted in substantial variation in total phenol, rutin, anthocyanin contents and antioxidant activity but results did not follow a chronological trend. Recently a number of breeding programs focusing on the bioactive phytochemicals, so called functional breeding, have started for some plants and vegetables. This study provides useful information on the differences of phenolic profile and antioxidant activity of asparagus varieties for functional breeding of asparagus. Antioxidant activity of asparagus is positively correlated with total phenolic and rutin content, suggesting that phenols are mainly responsible for antioxidant activity.

Rutin and chlorogenic acid were identified as the major phenols and antiradical compounds in both green and purple asparagus varieties. A unique compound was detected in 'Purple Passion' asparagus but it could not be identified. However this compound did not show antiradical activity.

FUTURE RESEARCH

The research on varietal and seasonal differences in phenolic content and antioxidant activity of asparagus should be continued in the following years to obtain more accurate results. The temperature and solar radiation values can be recorded at the field to determine the effect of seasonal conditions on rutin content and antioxidant activity.

Continuing research should also include the identification of unknown compounds. The first peak in both green and purple asparagus and the fourth peak in the purple asparagus chromatogram can be identified using more sophisticated methods. Peak 1 and chlorogenic acid were suggested to have similar structures such as isomers. These peaks could be collected and structural differences can be determined using mass spectrometry, NMR or infrared spectroscopy. Different extraction methods can be applied since multi-step purification caused some losses in the phenolic content. The same solvent can be used for both spectrophotometric and chromatographic assays, so that results can be compared more accurately. The anthocyanin fraction of extracts can be analyzed by HPLC/DAD to determine the specific anthocyanins in asparagus. This fraction can also be analyzed using the HPLC-DPPH method to compare the antiradical activity of anthocyanins against other phenolics, rutin and chlorogenic acid.

The present study report the statistical differences among asparagus varieties by *in vitro* assays. The bioavailability of asparagus antioxidants in biological systems needs to be measured by *in vivo* test. Therefore, practical differences among cultivars can be determined.

APPENDIX

The antioxidant capacities of the same asparagus varieties were also measured using TEAC assay by another researcher in our laboratory. Results showed that hydrophilic fraction TEAC values were noticeably larger than the lipophilic fraction. Hydrophilic TEAC values expressed as μM Trolox equivalent (TE)/g of DW asparagus are given in **Table 1.** The highest activity was obtained for 'Guelph Millenium' with 69.10 μM TE/g of DW followed by 'Jersey Knight', 'Jersey Supreme', 'Jersey Giant', 'Jersey Deluxe' and 'Morehouse Select'. No significant difference was found among those varieties. 'Syn 4' exhibited significantly lower activities while 'UC 157' and 'Purple Passion' showed the lowest (49.38 and 49.37 μM TE/g of DW, respectively).

During the course of the study, asparagus varieties from Mexico, California and Peru were purchased from local supermarket (Pullman, WA). These samples were analyzed spectrophotometrically for total phenolic, rutin and anthocyanin content as well as antioxidant activities. Their phenolic profiles were determined by HPLC-DAD. Origin of the samples, date of purchase and results of the analysis are given in **Table 2, 3** and **4**. Those samples mainly demonstrated significantly lower results when compared to Washington asparagus. However, it is not possible to make a valid comparison between samples because imported asparagus samples may have been on the shelf for longer times while Washington asparagus were kept frozen since the day they were harvested.

Table 1. ABTS radical scavenging activities of nine asparagus varieties at three different harvest dates in 2006.

Variety	1st harvest	2nd harvest	3rd harvest	Mean ± std[1]
Jersey Knight	62.97 ± 8.32	65.85 ± 6.29	68.94 ± 1.18	**65.92 ± 2.99**[a]
Guelph Millenium	68.76 ± 3.14	57.14 ± 8.27	81.39 ± 9.36	**69.10 ±12.13**[a]
Jersey Deluxe	72.88 ± 6.76	58.57 ± 3.08	69.73 ± 3.09	**67.06 ± 7.52**[a]
Jersey Supreme	67.42 ± 1.67	59.41 ± 2.17	75.70 ± 8.18	**67.51 ± 8.15**[a]
Morehouse Select	67.04 ± 9.65	63.31 ± 1.09	69.30 ± 3.04	**66.55 ± 3.02**[a]
Jersey Giant	59.86 ± 2.26	64.59 ± 2.46	77.36 ± 2.66	**67.27 ± 9.05**[a]
Syn 4	48.83 ± 3.83	60.78 ± 1.29	65.09 ± 4.93	**58.23 ± 8.42**[b]
Purple Passion	37.63 ± 0.36	44.47 ± 6.52	66.00 ± 4.30	**49.37 ± 14.81**[c]
UC 157	46.76 ± 7.46	45.97 ± 1.07	55.40 ± 5.75	**49.38 ± 5.23**[c]

Values are expressed as μM TE/g dry weight asparagus and represent means ± SD (n=3)

[1]Means with different letters indicate significant differences ($P \leq 0.05$) by Tukey's test

Table 2. Total phenolic, rutin content and antioxidant activity of imported asparagus samples

Origin of sample	Purchase date	Total Phenolics[1]	Rutin Contents[2]	Antioxidant Activity[3]
Northern Mexico	March 3 2006	25.96 ± 0.66	9.29 ± 0.87	9.88 ± 0.34
Southern California	March 16 2006	28.78 ± 0.27	9.96 ± 0.18	9.06 ± 0.10
Central California	April 4 2006	22.75 ± 1.37	4.78 ± 0.68	6.94 ± 0.16
Central Mexico	August 3 2006	37.10 ± 1.24	13.28 ± 0.71	20.64 ± 2.72
Peru	August 4 2006	29.88 ± 1.35	8.16 ± 0.12	17.22 ± 1.16

[1] Total phenolic content were measured by Folin-Ciocalteu method. Values are expressed as mg rutin equivalent/g dry weight asparagus and represent means ± SD (n=2)

[2] Rutin content were measured colorimetric $AlCl_3$ method. Values are expressed as mg rutin/g dry weight asparagus and represent means ± SD (n=2)

[3] Antioxidant activities were measured by DPPH radical scavenging activity. Values are expressed as mg rutin equivalent/g dry weight asparagus and represent means ± SD (n=2)

Table 3. Total anthocyanins, rutin and chlorogenic acid content of imported asparagus samples

Origin of sample	Purchase date	Total Anthocyanins[1]	Chlorogenic Acid Content[2]	Rutin Content[3]
Northern Mexico	March 3 2006	0.16 ± 0.02	0.53 ± 0.36	1.47 ± 0.29
Southern California	March 16 2006	0.21 ± 0.02	0.50 ± 0.04	1.67 ± 0.13
Central California	April 4 2006	0.18 ± 0.02	0.44 ± 0.06	0.86 ± 0.17
Central Mexico	August 3 2006	0.18 ± 0.03	0.73 ± 0.05	1.88 ± 0.04
Peru	August 4 2006	0.40 ± 0.02	0.53 ± 0.10	1.63 ± 1.10

[1] Total anthocyanins were measured spectrophotometrically. Values are expressed as mg total anthocyanins/g dry weight asparagus and represent means ± SD (n=2)

[2] Chlorogenic acid contents were measured by HPLC-DAD. Values are expressed as mg chlorogenic acid/g dry weight asparagus and represent means ± SD (n=2)

[3] Rutin contents were measured by HPLC-DAD. Values are expressed as mg rutin/g dry weight asparagus and represent means ± SD (n=2)

Table 4. Trolox equivalent antioxidant activity of imported asparagus samples measured by ABTS and DPPH radical scavenging assays.

Origin of sample	Purchase date	DPPH[1]	ABTS[2]
Northern Mexico	March 3 2006	53.71 ± 1.31	36.24 ± 0.69
Southern California	March 16 2006	49.63 ± 0.66	52.78 ± 3.51
Central California	April 4 2006	44.24 ± 0.20	52.67 ± 3.54
Central Mexico	August 3 2006	99.19 ± 10.91	76.62 ± 4.66
Peru	August 4 2006	85.84 ± 3.93	71.67 ± 0.99

[1] Values are expressed as μM TE/g dry weight asparagus and represent mean ± SD (n=2)

[2] Values are expressed as μM TE /g dry weight asparagus and represent mean ± SD (n=2)