ABTSTRACT

CHOONG, TEOW. Antioxidant activity and bioactive compounds of sweetpotatoes. (Under the direction of Dr. Van-Den Truong)

Sweetpotatoes are rich in dietary fiber, minerals, vitamins and bioactive compounds such as β-carotene, phenolic acids and anthocyanins which provide sweetpotato clones with distinctive flesh colors (cream, yellow, orange and purple). Limited studies have been conducted on the total antioxidant capacity of various sweetpotato types in relation to their phytochemical content and composition. This study aimed to determine the free radical scavenging activity of sweetpotatoes using several common methods, to assess the correlation among the antioxidant assays, and to determine the relationship between the antioxidant activity and the phenolic, anthocyanin and β-carotene content in breeding lines. Oxygen radical absorbance capacity (ORAC), 2,2-diphynyl-1-piicrylhydrazyl (DPPH) and 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) methods were used to evaluate antioxidant activity . Total phenolics were measured using the Folin-Ciocalteau method, total anthocyanins by the pH-differential method, and β-carotene by HPLC. Results indicated that dark purple-fleshed sweetpotatoes had high antioxidant activity, total phenolic and anthocyanin contents. The total phenolic and β-carotene contents were highly correlated with the antioxidant activity of the hydrophilic and lipophilic extracts, respectively. There were good correlations between ORAC and ABTS, and ORAC and DPPH suggesting that these methods have similar predictive capacity for sweetpotato antioxidant capacity.

With high antioxidant capacity, the purple-fleshed sweetpotatoes have good potential for health food markets. Characterization of the pigments in a genotypically diverse group of purple-fleshed clones for physiological functionality and polyphenolic compounds associated with the color of the derived progeny is important for breeding program as well as suitability for processing. The results showed that the anthocyanin and phenolic contents as well as antioxidant activities greatly varied among the cultivars. Heat treatment resulted in significant increase in the polyphenolic content and composition of the extracts. Based on visual color measurement, the clones were categorized as a red-dominant group (peonidin-rich). However, HPLC analysis revealed that the ratios of peonidin and cyanidin varied widely among the samples with several genotypes in a cyanidin-rich group. The genotypic diversity of purple-fleshed sweetpotatoes may make it possible to utilize them in a variety of food and nutraceutical applications.

ANTIOXIDANT ACTIVITY AND BIOACTIVE COMPOUNDS OF SWEETPOTATOES

By

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DEDICATION

Dedicated to my loving mother and father.

BIOGRAPHY

Choong Teow was born on May 23, 1978 in Penang, Malaysia to Lee Seong Teow and Chuan Chuan Chuah. He has an elder brother, Choong Hor Teow. After graduation from high school in Malaysia, he attended Warren Wilson College (Asheville, N.C.) and obtained a B.S. degree in Chemistry in 2003. He then began working towards a M.S. degree in Food Science at NCSU under the direction of Dr. Den Truong. During the summer he had the opportunity to do an internship at a pharmaceutical company in Sanford, N.C., and he will continue working there after graduation.

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Literature Review

I. Sweetpotato Origin and Production

Since the 16th century, sweetpotatoes have been a traditional food in Asia primarily in China. Sweetpotatoes were one of the primary food commodities in China due to the well adaptability of the crop to the environmental conditions in that country. Compared to other crops, the storage root of sweetpotato is able to grow at an accelerating rate in various environmental conditions, thus multiplying in quantity in a short period of time. For that reason, sweetpotato spread throughout Asia, Africa, and Latin America during the 17th and 18th centuries. Since then, sweetpotato has become one of the most grown crops in Asia, Africa, and America and so did the demand for sweetpotato among consumers.

Globally, sweetpotato is ranked as the seventh most important food commodity after wheat, rice, maize, potato, barley and cassava. Among all the root crops that are consumed, sweetpotato ranks second only to the potato in economic importance (Horton and Rama, 1988). In the United States, sweetpotatoes are also a popular commodity grown primarily in the southern states such as North Carolina, Louisiana, Mississippi and Texas. According to the Food and Agriculture Organization of United Nations, 98% of sweetpotato production occurs in developing countries in 2004. The United States produces less than 1% of the total world sweetpotato crop. More than 142 million tons of the roots were produced globally in the year 2000. It was estimated that the production of sweetpotato in the United States was approximately 743,000 ton (metric) in 2004. North Carolina was the top producer of sweetpotato in 2004, with a yield of 312,000 tons (metric). Since statistics have been kept, North

Carolina has always been the number one state in the production of sweetpotatoes in the United States, supplying more than 40% of the total sweetpotato production in this country, followed by Louisiana and California (ERS, 2003) in sweetpotato production. In recent years, interest in incorporating sweetpotato as an ingredient in processed foods has been increased in the U.S. due to the recognition in its high nutritional value.

A. Diversity of Sweetpotato and its Composition

Sweetpotato is a nutritionally rich crop. Various types of nutrients are found in sweetpotato, including antioxidants, vitamins (B_1, B_2, C and E), minerals (calcium, magnesium, potassium and zinc), dietary fiber, protein and non-fibrous carbohydrates (Suda et al., 1999; Woolfe, 1992).

The major antioxidants in the sweetpotato storage root are phenolic acids, anthocyanins, and carotenoids. These bioactive compounds act as free radical scavengers and also contribute to the distinctive colors of sweetpotatoes. Approximately 80 percent of the dry matter of sweetpotatoes is carbohydrate. Most of the carbohydrate is starch. There is a substantial amount of soluble sugar, cellulose, pectin, and hemicellulose. The protein concentration is relatively low and is only about 5% of the dry matter in sweetpotato. Lipid consists of approximately1.2-2.7 percent of the total fresh weight. It is composed of neutral lipid, glycolipids, and phospholipids, organic acids including malic acid, quinic acid, succinic acid, and citric acid. They function as intermediates in metabolism and contribute to the taste of the storage roots (Kays et al., 1992). Vitamins such as vitamin C and E also present in the root. Both vitamin C and vitamin E are

important antioxidants that break the chain reaction of free radicals. Unlike vitamin C, vitamin E is fat soluble, therefore it works in lipid tissues (Combs, 1998). Amylases are technologically important enzymes in the sweetpotato that break down starch to shorter chain products (Woolfe, 1992).

In the United States, the sweetpotato breeding programs of USDA-ARS and several universities have developed advanced clones and new cultivars of sweetpotatoes since they started a collaborative program in the early 1970s. As a result, many different cultivars of sweetpotato with different desired traits were developed. The newly developed clones offered many advantages including resistance to insects and diseases. High quality sweetpotatoes with a range of flesh and skin colors and higher level of phytochemicals, such as ascorbic acid, α-tocopherol, flavones, β-carotene and anthocyanins were derived from the breeding programs (Jones and Bouwkamp, 1992). Variations in the content of carotenoids and anthocyanin pigments are responsible for the variation in the flesh color of sweetpotatoes from white, to yellow, orange and purple. Takahata at al. (1993) reported that some orange-fleshed sweetpotato cultivars contain more β-carotene than carrots.

B. Phytochemicals of Sweetpotato

Sweetpotatoes contain various types of bioactive compounds such as phenolic compounds and carotenoids. Phenolic compounds that are found in sweetpotato include phenolic acids and anthocyanins, which are predominant in purple-flesh sweetpotatoes. Carotenoids, such as α-carotene, β-carotene, and β-

cryptoxanthin are predominant in orange-fleshed sweetpotatoes (Kay et al., 1992; Woolfe, 1992; Yoshinaga et al., 1999).

Phenolic Compounds

Phenolic compounds are natural antioxidants having an aromatic ring with one or more hydroxyl groups. Numerous types of phenolics are found in nature, including simple phenol, phenylproponoids, benzoic acid derivatives, flavonoids, stilbenes, tannins, lignans and lignins. Phenolic compounds in fruits and vegetables are the secondary metabolites in plants that are derived from the metabolism phenylalanine and tyrosine (Van Sumere, 1989).

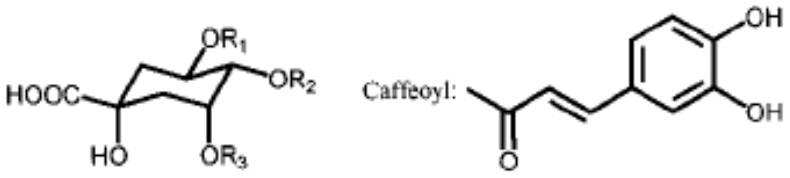
Phenolic compounds play a crucial role in the growth and reproduction of plants, and also act as antifeedants and antipathogens (Butler, 1992). These compounds also act as a natural defense mechanism by inhibiting mutations in plants. Phenolics function as antibiotics, natural pesticides, signaling substances, protective substance against ultraviolet light, insulating materials to make cell walls impermeable to gas and water, and give structural stability to plants.

Phenolic compounds are synthesized in plant tissues. Therefore their occurrence in animal tissues is generally due to the ingestion of plant foods. However, many food industries have incorporated these compounds into food products to prevent lipid oxidation, which can cause off odor in lipid-rich food. Fruits and vegetables are excellent sources of phenolic compounds. Ehlenfeldt and Prior (2001) surveyed the phenolic content of different varieties of

blueberries, and they reported that the phenolic content of these berries were relatively high, ranging from 490 to 1990 mg gallic acid/kg fw.

The phenolic contents of the sweetpotatoes cultivated in the US ranged from 117 to 467 mg of chlorogenic acid equivalent/kg of fresh weight (Walter and Purcell, 1979). Chlorogenic acid is the major phenolic compound found in sweetpotatoes (Suda et al., 2002; Yoshinaga et al, 1999; Walter and Purcell, 1979).

In many countries, both the storage roots and leaves of sweetpotato are consumed (Nwinyi, 1992). Research in Japan indicated that the foliage of sweetpotato is a rich source of phenolic compounds (14.2 to 171 g of chlorogenic acid equivalents/kg of dry matter). Six different phenolic acids have been isolated and identified in the leaves of sweetpotatoes (Figure 1). Among them 3,5-tri-O-caffeoylquinic acid and 4,5-di-O-caffeoylquinic acid are the predominant compounds (Islam et al., 2002).



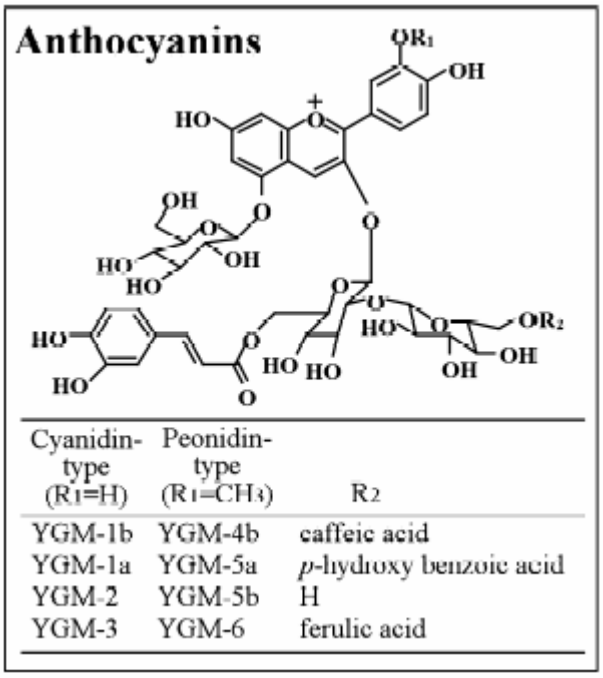
	R_1	R_2	R_3
3-*O*-caffeoylquinic acid:	caffeoyl	H	H
4,5-di-*O*-caffeoylquinic acid:	H	caffeoyl	caffeoyl
3,5-di-*O*-caffeoylquinic acid:	caffeoyl	H	caffeoyl
3,4-di-*O*-caffeoylquinic acid:	caffeoyl	caffeoyl	H
3,4,5-tri-*O*-caffeoylquinic acid:	caffeoyl	caffeoyl	caffeoyl

Figure 1: Phenolic compounds in sweetpotato (Islam et al., 2002)

Anthocyanins are phenolic compounds that are widely studied in fruits and vegetables. Similar to phenolic acids, anthocyanins have free radical scavenging

activity. Anthocyanins are water-soluble pigments that impart red, purple, and blue coloration to many fruits and vegetables. Anthocyanins are found abundantly in different kinds of berries including blueberry, cranberry, lingonberry and chokeberry. These berries have an anthocyanin contents of 120, 32, 45 and 428 mg/ 100g fwb, respectively (Zheng and Wang, 2003).

The anthocyanin pigments give purple-fleshed sweetpotatoes their distinctive colors. In Japan, the purple-fleshed cultivars, Yamagawamurasaki (YGM) and Ayamurasaki have been cultivated for use as a natural food colorant (Yoshinaga et al., 1995). There are eight types of anthocyanins in purple-fleshed sweetpotatoes, which are categorized into two major groups: peonidin anthocyanins and cyanidin anthocyanins. The difference between the two groups is the presence or absence of a methyl group at the 3'-position of the aromatic B-ring. Theses pigments can be either mono- or di-acylated forms of cyanidin (YGM-1a, -1b, -2 and -3) and peonidin (YGM-4b, -5a, -5b and -6) (Goda et al., 1997) as shown in Figure 2.



Cyanidin-type (R1=H)	Peonidin-type (R1=CH3)	R2
YGM-1b	YGM-4b	caffeic acid
YGM-1a	YGM-5a	*p*-hydroxy benzoic acid
YGM-2	YGM-5b	H
YGM-3	YGM-6	ferulic acid

Fig. 2: Chemical Structures of Peonidin and Cyanidin (Goda, 1997)

Among the 8 major anthocyanins in the purple-fleshed sweetpotatoes, two of them (YGM-2 and YGM-5b) are mono-acylated by caffeic acid, and the others are di-acylated by caffeic acid alone (YGM-1b and YGM-4b), caffeic acid and p-hydroxybenzoic acid (YGM-1a and YGM-5a), or caffeic acid and ferulic acid (YGM-3 and YGM-6). One common characteristic among all the types of anthocyanins in purple-fleshed sweetpotatoes is that they are bound to at least one caffeoyl group which makes them very good free radical scavengers.

The peonidin: cyanidin ratio in purple-fleshed sweetpotato affects the color, of the raw and cooked roots. Studies have indicated that purple-fleshed sweetpotatoes that have a higher ratio of peonidin to cyanidin also have a greater degree of redness. On the other hand, sweetpotatoes rich in cyanidin have a greater degree of blueness (Yoshinaga et al., 1999).

Anthocyanin pigments are pH sensitive and therefore undergo reversible structural transformations, with a change in pH that result in major changes in the absorbance spectra. The colored oxonium form predominates at pH 1.0 and the colorless hemiketal is the major form at pH 4.5. The degree of color stability of anthocyanins after heating and ultraviolet light irradiation is associated with the acylated anthocyanins species in the pigment (Hayashi et al., 1996). Due to the presence of a high proportion of acylated anthocyanins, purple-fleshed sweetpotato anthocyanins are more stable than to anthocyanins with low levels of acylation, such as those found in strawberry, raspberry, apple, and soybean with black seed coats (Hayashi et al., 1996).

Carotenoids

Carotenoids are compounds that consist of eight isoprenoid units. These isoprenoids units are easily recognizable as they are joined in a head-to-tail pattern, but the order is inverted at the center of the structure (Figure 3.0). Carotenoids can be categorized into two major groups: carotenes and oxycarotenoids. The difference between these two groups is that in addition to having hydrogen and carbon atoms, oxycarotenoids also contain oxygen groups. Various carotenoids, including α-carotene, β-carotene, and β-cryptoxanthin (Figure 3.0), can be transformed into retinal (vitamin A) by mammals. On a molar basis β-carotene has the most provitamin A activity among the carotenoids that can be converted to vitamin A.

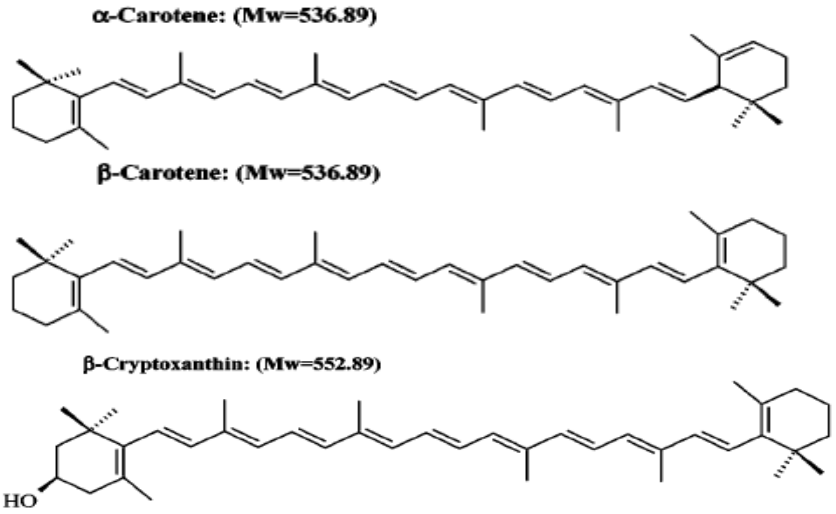


Figure 3: Structure of Carotenoids (O'Neal, 1992)

The dark orange characteristic colorings of plants are derived from β-carotene, which is found abundantly in most fruits and vegetables. The cream and orange color of sweetpotatoes is due to β-carotene. In general, white-fleshed sweetpotatoes contain very little to no β-carotene. In addition to β-carotene, other types of carotenoids are also found in orange-fleshed sweetpotato. These include alpha-, gamma- and zeta-carotenes, phytoene, phytofluene, beta-carotene-epoxide, hydroxy-zeta-carotenes and beta-carotene furanoxide (Purcell and Walter, 1968). However, theses carotenoids normally consist of less than 1% of the total carotenoids found in orange-fleshed sweetpotato. Woolfe (1992) found that 86.4-89.0% of the carotenoids in yellow and orange sweetpotatoes were β-carotene.

Kays et al. (1993) analyzed β-carotene content of various sweetpotato cultivars. They reported that the highest β-carotene content among all the sweetpotatoes analyzed was 190 ug/g dw. Other provitamin A carotenoids such

as α-carotene, and β-cryptoxanthin were not found in the storage roots. In Japan the β-carotene contents of sweetpotato with different colors have been studied using HPLC. The results indicated that orange-fleshed sweetpotatoes had the highest β-carotene content, whereas the purple-fleshed sweetpotatoes had traces or undetectable amounts of β-carotene. The β-carotene content of various types of sweetpotato cultivated in the United State was analyzed. Those cultivars included Jewel, Regal, Centennial and Resisto had 58, 60, 76 and 48 μg/g dw respectively (Kays et al., 1993). Huang et al. (1999), also reported that orange-fleshed sweetpotato had relatively high β-carotene content (131μg/g fw) as compared to sweetpotato of other flesh colors.

C. Potential Health Benefits of Sweetpotatoes

Dorogokupla and Zdavoohkr (1977) found that mice that were fed with diet rich in β-carotene had slower rates of cancer cell growth compared to a placebo diet (Dorogokupla and Zdavookhr, 1977). There is also evidence indicating that there is a higher correlation between high β-carotene intake in diet and lower incidences of cancer, especially lung cancer (Van Poppel and Goldbohm, 1995; Grassmann et al., 2002)

Dietary phenolic compounds have been recognized by the board of nutrition in the United States as an important health-promoting agent (Jones and Bouwkamp, 1992). In addition to being a potent antioxidants, phenolic compounds also have other functions. Phenolic compounds are able to bind to cellular receptors and transporters, which consequently influence gene expression, cell signaling and cell adhesion (Scalbert et al., 2003). The capability

of phenolic compounds to scavenge free radicals is the primary mechanism where phenolic compounds protect the cells from free radical attack.

There has been increasing interest in purple-fleshed sweetpotato lately due to the high anthocyanins content. Diacylated anthocyanins isolated from the storage roots of a typical breed of purple-fleshed sweetpotato, Ayamurasaki, were identified to possess a postprandial antihyperglycemic (antidiabetic) effect in rat through retardation of maltase activity. In relation to that, the development of antihyperglycemic food from purple-fleshed sweetpotato has been of great interest lately (Matsui et al., 2002). Anthocyanins and phenolic acids have also been reported to possess potential cancer chemopreventive effects. Because of the protective effect of anthocyanins against chronic diseases, different types of food processing methods have been designed to preserve the bioavailability of anthocyanins in sweetpotatoes (Heins et al., 2001). The “Ayamurasaki” extract has been observed in vitro to be a potent antioxidant or radical scavenger (Furata, 1998), antimutagen (Yoshimoto et al., 1999), and angiotensin I-converting enzyme inhibitor (Suda et al., 1999). In a rat study, purple-fleshed “Ayamurasaki” juice exhibited an ameliorative effect against carbon tetrachloride-induced liver injury (Suda et al., 1997). Purple-fleshed sweetpotato cultivars have a higher radical-scavenging or antioxidative activity than those with white, yellow, or orange flesh (Furata et al., 1998). Polyacylated anthocyanins were identified as the leading scavengers among all bioactive compounds found in purple-fleshed sweetpotato (Odaka et al, 1992; Goda et al. 1997; Yoshimoto et al, 1999).

Since the discovery of the physiological functionality of sweetpotatoes and their predominant anthocyanins pigments, the food industry, primarily in Japan, has incorporated sweetpotato as ingredients in processed food products. The demand for food products made from purple-fleshed sweetpotato has increased all over Japan since consumers became aware of the health benefit of consuming bioactive compounds like anthocyanins. At present, the paste and flour from the “Ayamurasaki” have been used in Japan for making noodles, bread, jams, sweetpotato chips, confectionery, juice, alcoholic drinks and food dyes.

Studies have indicated that deficiency of β-carotene can result in vitamin A deficiency, lead to xerophthalmia, a disease that causes irreversible blindness. Millions of children from developing countries are affected by this disease due to malnutrition, vitamin A deficiency in particular (FAO, 1988).

The American Heart Association has invested time and money educating the public about the usefulness of antioxidants, which lead to reducing rate of heart disease in the United States. A major breakthrough in heart disease occurred in 1974 when aspirin was shown to slow platelet aggregation. Future heart disease research revolved around vitamin E, which indicated that regular consumption of vitamin E will decrease chances of heart disease. In 1991 it was observed that French people who as a nation typically eat fattier foods, suffered from less heart disease than did their American counterparts. It has been postulated that the wines, especially red wines, drunk during meals by the French, provided protection to their cardiovascular system through antioxidant

activity, similar to that of vitamin E (Renaud and de Lorgeril 1992). This is presently referred to as the “French Paradox” leading to increased interest in the antioxidative activity of colored fruits and vegetables.

II. Antioxidants

A. Mechanism of Antioxidation and Disease Prevention

Oxidation is a process where electrons are transfered from one atom to another, with the molecule losing an electron being oxidized. Free radicals are generated in the human body when oxidation occurs during aerobic respiration.

Reactive oxygen species (ROS) are the oxygen-centered free radicals. They exist in different forms such as superoxides (O2•-), peroxyls (ROO•), alkoxyls (RO•), hydroxyls (HO•), and nitric oxides (NO•) (Pietta, 2000). Free radicals are also derived from external sources such as cigarette smoke, air pollution, ultra-violet light and ionizing radiation. These free radicals not only cause premature aging and wrinkles, but they are also the primary cause of cancer and other chronic diseases. Fortunately, our body has adapted with the changes in the atmosphere over time to develop defense mechanisms to reduce the damage done by free radicals. Antioxidants are the main defense mechanism in the body acting as free radical scavengers (Pietta, 2000). Besides damaging the cells and caused severe diseases, ROS can also potentially causes oxidation in food products. The major concern is primarily lipid oxidation, which alter the organolaptic properties of food product by causing off-flavors, shortening shelf life and diminishing the sensory aspects of food.

Antioxidants produced within the body include dismutase, peroxidase, and catalase enzymes, as well as glutathione (GSH) and cytochrome P450 (Rimbach et al., 2005). Antioxidants from fruits and vegetables include phenolic compounds, anthocyanins, carotenoids; vitamin C and vitamin E. Synthetic antioxidants used as food additives include bytylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (THBHQ). Regardless of the sources of antioxidants, all the antioxidants have a similar function, which is to prevent damage done by free radicals.

There are three classes of antioxidant capabilities in the human body. The first is work done by enzymes to control initial free radical production. When oxygen is taken in and used during aerobic respiration, superoxide, hydrogen peroxide, and hydroxyl radicals are all commonly formed (Gutteridge and Halliwell, 1994). Catalase and dismutase enzymes decrease the formation of hydroxyl radicals (McCord and Fridovich 1969). Glutathione peroxidases remove all peroxides, including hydrogen peroxides (Foyer and Halliwell 1976). All the enzymes require metal co-factors to function. For example, dismutases require copper, zinc or manganese, depending on the pH (McDord and Fridovich, 1969) to function. On the other hand, catalases require iron as a co-factor, and glutathione peroxidases may require selenium (Halliwell and Gutteridge 1989).

The second class of antioxidants come from the diet. Dietary antioxidants are found in various fruits and vegetables including sweetpotatoes. Dietary antioxidants are crucial in the human body because they are capable of ending the chain reaction of free radicals through proton donation.

The third class of antioxidants is replenishers. The source of protons comes from structures that can readily donate a proton, while remaining stable so as not to become a free radical. Examples of replenishers include carotenoids, flavonoids coenzyme Q, and glutathione. Both β-carotene and coenzyme Q work in lipid material and have a synergistic relationship with vitamin E (Ernster and Nelson 1981). Glutathione and flavonoids are tied more to the aqueous environment where they can work both as proton donors to free radicals, and act to replenish vitamin C. Uric acid also contributes protons to antioxidants (Davies et al., 1986).

Other methods to control the impact of free radicals include the body's handling of metals, and its ability to correct damage done through oxidation. When metals such as copper, iron, and manganese are not being used in the mitochondria to turn superoxide back into oxygen, or as co-factors to enzymes they are always bound to a carrier (eg. iron has ferritin or transferrin). They would be far too catalytic in the formation of new free radicals if not bound because of their ability to donate and accept an electron.

Antioxidants have the ability to repair damage done by free radicals and this is thought to reduce cancer risk and aging. Repair of DNA is done by glutathione where this antioxidant donates protons to mildly damaged DNA. On the other hand, the repair of lipids is done by phopholipase enzymes, which catalyze the cleavage of peroxidized fatty acid side chains from the membrane, and replaces them with new, undamaged fatty acids (Halliwell and Gutteridge,

1994). In cases where free radical attack is uncontrollable and cell damage cannot be repaired, the end result would be growth of cancerous cells.

The Hydrogen Atom Transfer mechanism measures the ability of an antioxidant to quench free radicals by donating hydrogen. The reaction mechanism is shown below:

$$X^{\bullet} + AH \rightarrow XH + A^{\bullet}$$

This is the most common reaction of most antioxidants. Relative reactivity in the Hydrogen Atom Transfer method is determined by the bond dissociation energy of the H-donating group in the potential antioxidant, dominating for compounds with ΔBDE of approximately -10 kcal/mol and ionization potential (ΔIP) of less than -36 kcal/mol (Wright et al., 2001). Antioxidant capacity measurements are based on competition kinetics. The Hydrogen Atom Transfer mechanism is pH and solvent dependent. In addition, the reaction is rapid. Interference of a reducing agent such as metals can cause misleadingly higher than actual reactivity.

The Single Electron Transfer method measures the ability of antioxidant to transfer one electron to reduce any compound, including free radicals, metals and carbonyls (Wright et al., 2001):

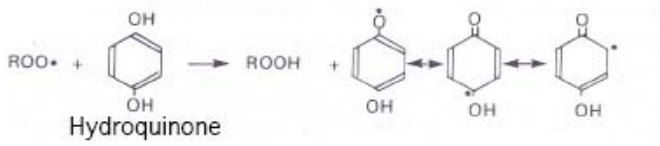
$$X^{\bullet} + AH \rightarrow X^{-} + AH^{\bullet+}$$

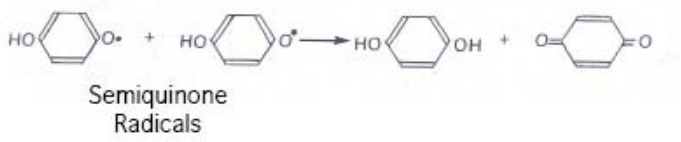
$$AH^{\bullet+} \xleftrightarrow{H_2O} A^{\bullet} + H_3O^{+}$$

$$X^{-} + H_3O^{+} \rightarrow XH + H_2O$$

$$M(III) + AH \rightarrow AH^{+} + M(II)$$

The first step of antioxidant activity involving phenolic compounds is the hydrogen atom transfer mechanism. The following example illustrates the mechanism by which hydroquinone, classified as a type of phenolic compound, is capable of breaking the chain reactions of free radicals by donating a proton / hydrogen ions from the two hydroxyl groups attached to the phenolic ring structure. Once hydroquinone reacts with free radicals such as peroxy radicals, it forms a stable semiquinone resonance hybrid (Nawar, 1985) as shown in the diagram below:





The stable semiquinone radicals that are produced further react with other peroxy radicals to inhibit more free radicals. In addition, semiquinones can also react with one another as shown in the second equation above. In general, flavonoids and phenolic compounds have the same mechanism of donating hydrogen ions while maintaining a stable structure. The reaction involves quenching of peroxy radical (ROO•) by the donation of a hydrogen atom from an aromatic ring antioxidant (ArOH) stabilizing the free radicals. The aromatic radicals (ArO•) remain stable by aromatic ring stabilization and hydroperoxide is produced. The general equation is shown below:

$$ROO\bullet + ArOH \rightarrow ArO\bullet + ROOH$$

Consequently, antioxidant follows a single electron transfer pathway for antioxidant activity, which inhibit propagation of peroxyl radical (ROO•) by hydrogen atom transfer. The single electron transfer pathway is shown below:

$$ROO\bullet + ArOH \rightarrow ROO^- + ArOH^{+}\bullet$$
$$ROO^- + ArOH^{+}\bullet \rightarrow ArO\bullet + ROOH$$

Antioxidant (ArOH) donates an electron to stabilize the peroxy radical (ROO•) producing a peroxy radical ROO- that is negatively charged. As the aromatic ring is now positively charged, it attracts the negatively charged peroxyl radical (ROO_{-}) and a proton (H_{+}) is donated to quench the charged hydroperoxide. There are various types of synthetic and natural antioxidant added as part of food ingredients. Studies have shown that there are approximately over 800 different types of phenolic compounds found in fruits and vegetable that are potential dietary antioxidants (Pietta, 2000).

B. Extraction of Antioxidants

Many different methods have been established to isolate and extract antioxidant compounds from plant materials. When choosing an extraction method, the major priority is to preserve the bioactive compounds and extract most if not all the desired compounds. Wet extractions involve solid material in direct contact with a liquid solvent (Houghton and Raman 1998). During the extraction, organic solvents diffuse into the solid material and solubilize

compounds with similar polarity. The nature of the solvent used will determine the types of chemicals likely extracted from the plant. Organic solvents for extractions include polar solvents such as water, ethanol, methanol and acetone, to non-polar solvents such as dichloromethane (DCM) and hexane.

Antioxidants can be physically classified by their solubility into two groups (i) hydrophilic antioxidants, such as the majority of polyphenolic compounds and vitamin C and (ii) lipophilic antioxidants, mainly carotenoids and vitamin E.

Similar to hydrophilic antioxidants, lipophilic antioxidants play an important role in a wide spectrum of biochemical and physiological processes. The main interest is their optimal antioxidant activity in vitro and in vivo. Unlike hydrophilic antioxidants, which do not accumulate in the body and are excreted in the urine, lipophilic antioxidants penetrate the lipoprotein cell membrane more easily and therefore reach a higher level of bioavailability.

In most of the studies on antioxidant activities in various food commodities, polar solvents such as ethanol, methanol, acetone and water have been used, and consequently, the extracts contain mainly the hydrophilic components. Therefore, the antioxidant activities reported do not reflect the total antioxidant capacity of the food materials. It is necessary to obtain both lipophilic and hydrophilic extracts for measuring the total antioxidant activity in foods. However, the methods available for measuring antioxidant activity in food commodities such as oxygen radical absorbance capacity (ORAC) and other methods can only be conducted in aqueous systems. Huang and co-workers (2002) overcame this obstacle by introducing randomly methylated β-cyclodextrin

(RMCD) as a molecular host to enhance the solubility of lipophilic antioxidants in aqueous solution allowing the assay for antioxidant activity of lipophilic extracts (Huang et al., 2002). The technique involves extracting lipophilic antioxidants with hexane, followed by incorporating lipophilic compounds or extracts into a 7% (w/v) randomly methylated β-cyclodextrin in acetone:water (1:1). The use of cyclodextrin has been increasingly popular for enhancing the solubility of fat soluble compounds in an aqueous environment in pharmaceutical and food industries (Szente et al., 1998).

Prior and co-workers (2004) evaluated the total antioxidant activity of 28 common foods including fruits, vegetables, nuts, dried fruits, sweetpotato and rice bran. They sampled from different regions of US that were harvested during two different seasons. The results indicated that all the fruits and vegetables, except avocado and raspberry, had very low lipophilic antioxidant activity, with most values being less than 5% of the total antioxidant activity. In general, the hydrophilic fraction of most fruits and vegetable had a much higher antioxidant activity than the lipophilic fractions. Cranberry and lowbush blueberry had one of the highest hydrophilic antioxidant activity (Wu et al., 2004). Spinach, black beans and broccoli had one of the highest lipophilic antioxidant activities among all the vegetables 4.20, 4.47 and 1.72 μM TE/g fw. These values were reported in term of micro molar trolox equivalent per gram fresh weight. Wu et al., (2004) suggested that green pigments of vegetables or the lipophilic components associated with these pigments in the samples may be responsible for the high lipophilic antioxidant activity. The antioxidant activities obtained by Wu et al.,

(2004), which include the hydrophilic, lipophilic and total antioxidant activity of sweetpotato were 0.44, 8.58 and 9.02 μM TE/g fw respectively. For comparison, red potato had 0.28, 10.60 and 10.98 μM TE/g fw of hydrophilic, lipophilic and total antioxidant activities respectively. It is likely that the antioxidant activities of sweetpotatoes vary with cultivars with varying flesh colors.

For most fruits and vegetables, the total antioxidant activity is paralleled to the hydrophilic antioxidant as the hydrophilic antioxidant consisted of approximately 90% of the total antioxidant activity. Prior et al. (2005) also indicated that, there are several factors that may impact the antioxidant activity of foods, this include genetics, harvest season, geographic and environmental conditions. In addition, the method used to analyze the antioxidant activity is a primary factor that explains the deviation among different antioxidant analysis procedures. Different methods utilize different free radical generators and standards. Also the hydroxyl radicals used in the ORAC assay may not be compatible for the lipophilic antioxidant, which explains the low lipophilic antioxidant activity. There is a need to develop an assay method using free radicals that is more compatible for the lipophilic antioxidants.

C. Assay Methods for Antioxidant Activity

Several methods are used to evaluate antioxidant activities of natural compounds in foods or biological systems with varying results. The free radicals that are commonly used to assess antioxidant activity in vitro are 2,2'-azobis (3-ethyl-benzothiazoline-6-sulfonic acid)(ABTS) and 2,2-diphynyl-1-picrylhydrazyl (DPPH). Other methods that are commonly used in the United State are the

oxygen radical absorbance capacity (ORAC) assay and the ferric ion reducing antioxidant power (FRAP) assay. The ORAC analysis measures the ability of antioxidants to protect a fluorescent from damage by free radicals (Cao et al., 1993). FREP is an electron transfer-based antioxidant capacity assay. The oxidant, known as the probe, consists of a ferric salt, Fe(III)(TPTZ)2Cl3 (TPTZ) 2,4,6-tripyridyls-triazine). Once the antioxidant reacts with the oxidant, the absorbance of the assay is read at 594 nm and reported in term of FRAP value.

Oxygen Radical Absorbance Capacity (ORAC) Procedure

Oxygen Radical Absorbance Capity (ORAC) is a procedure widely used to measure antioxidant capacity in food, pharmaceutical, and cosmetic industries. This method was developed by Cao and co-workers (1993) and was further improved by Prior et al., (2003). The mechanism of ORAC involves measuring antioxidant scavenging activity against a peroxyl radical induced by 2, 2'-azobis(2-amidinopropane) dihydrochloride (AAPH) at 37 ^{0}C (Cao et al., 1993). Previously, β-phycoerythrin (B-PE), a protein isolated from *Porphyridium cruentum*, was used as the fluorescent probe. The loss of fluorescence of B-PE is an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of an antioxidant is measured by assessing the area under the fluorescence decay curve (AUC) of the sample as compared to that of the blank in which no antioxidant is present. The ORAC assay provides a complete assessment in which the inhibition time and inhibition degree are measured as the reaction goes to completion. However, one disadvantage of using B-PE is that it produces inconsistent results that vary within replicates and between days

due to variable reactivity of the peroxyl radical (Cao and Prior, 1999). B-PE is also not photostable, therefore, it can be photo-bleached, producing inaccurate results of antioxidant activity. B-PE also interacts with polyphenols in the samples due to nonspecific protein binding. Therefore, Prior et al. (2003) have introduced Fluorescein (FL) (3', 6'-dihydroxyspiro [isobenzofuran-1[3H],9'[9H]-xanthen]-3-one) to substitute for B-PE. Fluorescein is relatively stable as compared to B-PE, and it also has high sensitivity and is easily detectable by fluoremeters.

Fluorescein reacts readily with the peroxyl radicals (ROO•) generated by APPH (2,2'-Azobis(2-amidinopropane) that deteriorate fluorescein through a series of steps resulting in loss of fluorescence intensity (Ou, 2001) (Figure 4).

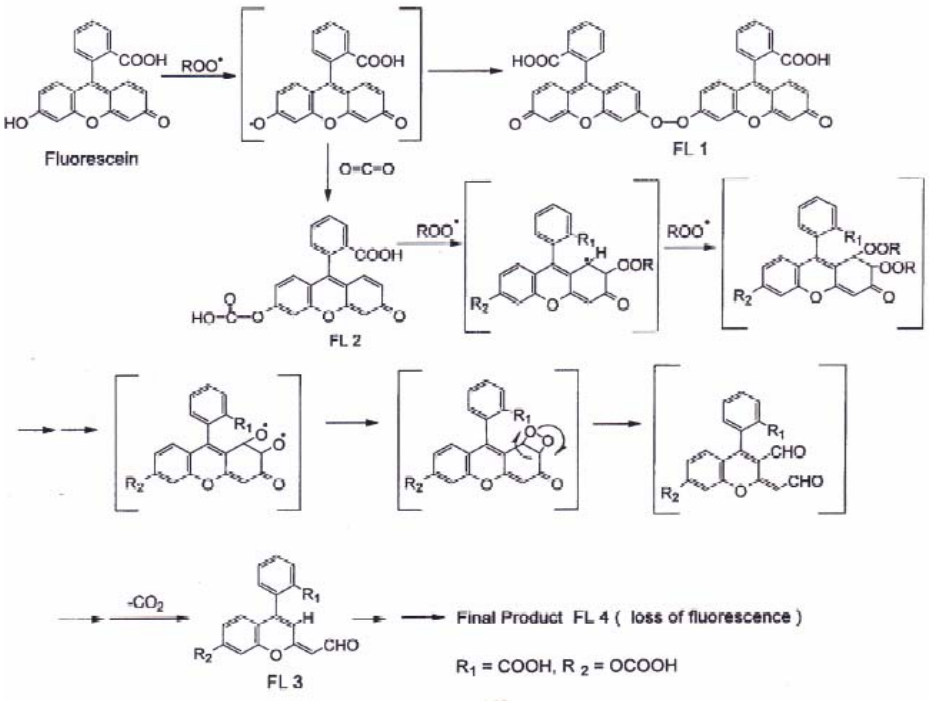


Figure 4. Fluorescein oxidation pathway in the presence of AAPH (Ou, 2001). FL 1 though 4 indicate the lost of fluorescence at four different stages.

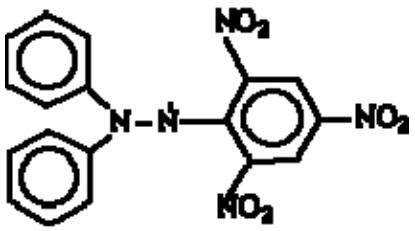
As mentioned above, the ORAC-FL assay is further extended to lipophilic antioxidants by using methylated β-cyclodextrin as a water solubility enhancer (Huang et. al. 2002).

2,2-Diphenyl-1-picrahydrazyl (DPPH) Method

The DPPH is a stable free radical with maximal absorption at 515 nm. It loses this absorption when reduced by an antioxidant or a free radical species (Brand-Williams et al., 1995). The reaction mechanism is shown in the diagram below where **AH** is the antioxidant and **R**$^{\cdot}$ is the free radical species:

DPPH$^{\cdot}$ **+ AH → DPPH-H + A**$^{\cdot}$
DPPH$^{\cdot}$ **+ R**$^{\cdot}$ **→ DPPH-R**

The structure of DPPH is shown in the diagram below:



The DPPH method allows a direct investigation of the ability for the extract or antioxidant to donate hydrogen and/or electrons to quench the DPPH radical, DPPH$^{\cdot}$. As the radical is quenched by antioxidants, the color of the solution changes from a deep purple to a light yellow and the absorbance at 515 nm decreases. The decrease in absorbance at a reaction time is used in determining the antioxidant activity of the tested substances with Trolox (2,5,7,8-tetramethychroman-2-carboxylic acid) as standard.

The DPPH method is widely used to determine antiradical/antioxidant activity of purified phenolic compounds as well as natural plant extracts. The

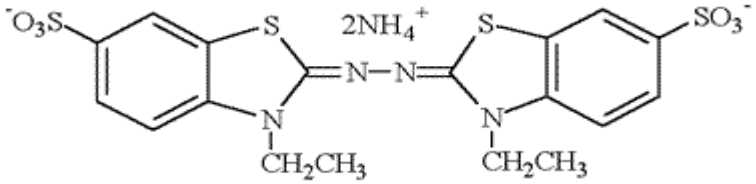
antioxidant activity of sweetpotato has been analyzed based on the DPPH method and the results suggested that the data was reproducible (Oki et al., 2002). Cevallos-Casals and Cisneros-Zevallos (2003) of Texas A&M University analyzed the antioxidant activity of red sweetpotato and Andean purple corn using the DPPH method. Their results were promising as they indicated that the antioxidant activity of sweetpotato was comparable to blueberries. This method also has good repeatability and is used frequently. However, the DPPH method also has its limitations. Bondet et al. (1997) reported that most phenolic antioxidants react slowly with DPPH, reaching a steady state in 1-6 hours or longer. This suggests that antioxidant activity using DPPH should be evaluated over time. Furthermore, color interference of DPPH with samples that contain anthocyanins leads to underestimation of antioxidant activity (Arnao, 2000).

Brand-Williams et al. (1995) found that certain antioxidant compounds have different reaction kinetics with DPPH•. Antioxidants such as BHT and protocatechuic acid did not reach steady state, or the reaction endpoint, until three and two hours respectively, whereas compounds like ascorbic acid, isoascorbic acid, and isoeugenol achieved steady state within one minute. At steady state, the DPPH• reaction has been shown to have a stoichiometric correlation with the quantity of antioxidant present. Caffeic acid, gentisic acid and gallic acid exhibited the highest antiradical activity with stoichiometry of 4.54, 5.6, and 6.25 reduced DPPH• molecules per molecule of antioxidant respectively while one molecule of phenol, ascorbic acid, α-tocopherol, and BHT reduced <1, 1.85, 2, and 2.63 molecules of DPPH• respectively (Brand-Williams et al., 1995).

There are three ways to explain the different efficiencies of monophenolic compounds in reducing one DPPH•. One mechanism involves the delocalization of an electron onto the parasubstituted OH group of the molecule prior to the donation of second hydrogen to reduce DPPH•. Another pathway involves the dimerization between two phenoxyl radicals in which two hydroxyl groups would be regenerated through an intramolecular transfer of H•, consequently reacting further with DPPH•. The final pathway stated is a complexation of the aryl radical directly with the DPPH•. All of these pathways depend on the structure of the antioxidants themselves, whether it will go through a dimerization between antioxidants or a direct complexation with DPPH• depends on the stability and reaction potential of the molecular structure (Brand-Williams, 1995).

2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) Method

ABTS is another commonly used free radical to access antioxidant activity in vitro. However, this free radical is foreign to biological systems. The structure of the ABTS free radical is shown in the diagram below:



The reaction mechanism of ABTS is similar to the DPPH reaction shown above except the free radical is generated by ABTS:

$ABTS^{\cdot}$ + AH → ABTS-H + $A^{\cdot}$
$ABTS^{\cdot}$ + $R^{\cdot}$ → ABTS-R

The ABTS assay measures the relative ability of antioxidant to scavenge the $ABTS^{\cdot+}$ generated in aqueous phase, as compared with a Trolox (water soluble vitamin E analogue) standard. In this analysis, the antioxidant capacity is tested by reacting a test compound with ABTS solution resulting in a decrease in the color of the solution. The $ABTS^{\cdot+}$ is generated by reacting with a strong oxidizing agent (e.g., potassium permanganate or potassium persulfate) with the ABTS salt. The reduction of the blue-green $ABTS^{\cdot+}$ radical by hydrogen-donating antioxidant is measured by the suppression of its characteristic long wave absorption spectrum (Miller and Rice-Evans, 1997). The method is usually expressed as Trolox equivalent antioxidant capacity (TEAC). The advantage of using this method is that it is rapid and can be used over a wide range of pH values (Arnao et al., 1999), in both aqueous and organic solvent systems. Based on the analysis of antioxidant activity of sorghum (*Sorghum bicolor*), the ABTS method is more cost effective and simpler to use compared to other method such as ORAC. In addition, the ABTS method also demonstrated similar predictive power as ORAC on sorghum antioxidant activity (Awika et al., 2003). It also has good repeatability and is simple to perform. However, this method has not been correlated with biological effects, therefore, it's actual relevance to in vivo antioxidant efficacy remains unknown (Awika et al., 2003). Pellergrini et al. (2003) demonstrated the time dependency of the ABTS assay. The results indicated that the reaction between ABTS and antioxidant compounds reacted completely at different rates depending on the antioxidant compound used.

Therefore, it was essential to select a time-point of measurement when using different food matrixes (Pellegrini et al., 2003).

III. Analysis of Phytochemicals

A. Analysis of polyphenols

There are several methods available for analyzing the total phenol content in plant foods including precipitation with heavy metals, precipitation by the addition of organic compounds, oxidation under controlled conditions, and formation of colored products with various chemical elements. The very first method developed, the Folin-Ciocalteu method (Folin and Ciocalteu, 1927) was developed in 1927 and it originated from chemical reagents used for tyrosine analysis in which oxidation of phenols by a molybdotungstate reagent yields a colored product at 745-750 nm:

$$Na_2WO_4/Na_2MoO_4 \rightarrow (\text{phenol}-MoW_{11}O_{40})^{-4}$$

$$\text{Mo(VI) (yellow)} + e^- \rightarrow \text{Mo(V) (blue)}$$

Among all the methods available today, only the Neubauer-Lawenthal method and the Folin-Dennis method are still in practice. The Neubauer-Lawenthal method was the official method of the Association of Analytical Communities (AOAC) for some time, and it was then replaced by the Folin-Denis method. The most recently established procedure for analyzing total phenolic content is the Folin-Ciocalteu reagent method, which has replaced the Folin-Denis reagent method (Singleton and Rossi, 1965). Therefore, total phenolic contents of sweetpotato extracts are determined using the Folin-Ciocalteu method.

The Folin-Ciocalteau procedure is based on the reductive power of aromatic hydroxyls with the phosphomolybdate complex of the Folin-Ciocalteu reagent (phosphomolybdate and phosphotungstate). This method measures the total hydroxyl groups of phenolic compounds. The absorbance is obtained by spectrophotometer reading at 725 nm. The concentration of the total phenolic content is determined by comparison with the optical density values of different concentrations of a standard phenolic compound, either gallic acid or chlorogenic acid, and is expressed in terms of gallic acid or chlorogenic acid equivalent (Sato et al., 1996).

The disadvantage of using the Folin-Ciocalteu reagent method is that it does not differentiate between different phenol types. Substances such as sugar, ascorbic acid, aromatic amines, sulfur oxide, iron and other compounds can interfere with the Folin-Ciocalteu assay. Therefore, correction for interfering substances should be made to accurately measure phenolic contents of samples. Non-phenolic substances and inorganic substances may also interact with Folin-Ciocalteu reagent, thus giving an inaccurate and higher than actual phenolic content (Prior et al., 2005).

Nevertheless, the Folin-Ciocalteu method correlated well with antioxidant capacity analysis procedures such as ORAC. Numerous researchers have indicated that there is a good relationship between ORAC analysis of the antioxidant activity of fruits with high phenolic content (Prior et al., 2005). Similarly, DPPH and ABTS procedures, which correlate well with the ORAC procedure, would also have good correlations with phenolic contents.

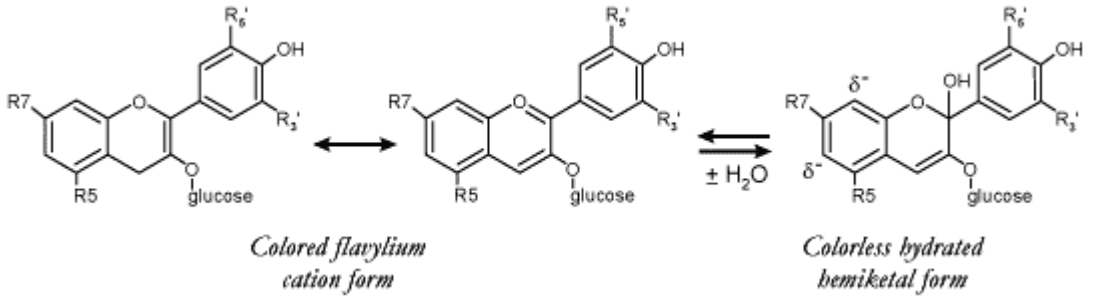
Cevallos-Casals and Cisneros-Zevallos (2003) analyzed the phenolic antioxidants of several fruits and vegetables with purple colors including purple corn, red-fleshed sweetpotato and blueberries. The results indicated that the purple corn and red-fleshed sweetpotato had a phenolic content of, 1756 and 945 mg/100g fw. The phenolic content of blueberry ranged from 292 to 672 mg/100g fw. The range of phenolic contents in blueberry could be due to different variety of berries and different level of maturity. Studies have shown that phenolic content is correlated to the maturity of the fruits, where phenolic content increases with aging of the fruits (Prior et al., 1998)

B. Analysis of Total Anthocyanins

There are several analytical methods for quantifying the total anthocyanins in fruits and vegetables. Among them are analysis using HPLC or by using anthocyanins as an indicator and titrating to a color change with a known quantity of base (Oomah and Mazza, 2000). The absorbance value of an alcoholic extract or the amount of base required to induce a color change is proportional to the total amount of anthocyanins present. When determining the total concentration of anthocyanins present, spectrophotometry is recommended over other techniques (Petri et al. 1997)

One of the widely used analyses of total anthocyanins is the pH-differential method. This method is based on the reversible change in the structure of anthocyanins and their absorbance spectra at different pH. All naturally occurring anthocyanins are in equilibrium between the color flavylium cation and the colorless hydrated form. However, the equilibrium occurs at lower

pH, around pH 1, the colored flavylium form predominates. At a higher pH, typically 4.5, the colorless hemiketal form is predominant. The structural transformation due to pH change concept was introduced by Sondheimer and Kertesz (Francis, 1989). They used pH values of 2.0 and 3.4 for analyses of strawberry jam (Francis, 1989). Since then others have adapted the idea and modified it to analyze different food commodities (Wrolstad et al., 1995). The structure flavylium and hemiketal are shown in the diagram below:



The pH-differential method is based on this reaction, which results in different absorbance at different pH values. This is a rapid and accurate method of determining the total anthocyanins, even in the presence of polymerized degraded pigments and other interfering compounds. To determine the total anthocyanins content, the absorbance is read at maximum visible wavelength and 700 nm, which allows for haze correction (Wrolstad and Guisti, 2001).

C. High Performance Liquid Chromatography Analysis and Color Value Measurement of Anthocyanins

Several analytical methods are available to analyze the composition of anthocyanins including high performance liquid chromatography (HPLC) and liquid chromatography mass spectrophotometry (LC-MS). HPLC is a technique used to separate the components of analysis and quantify them based on a

standard. Liquid chromatography mass spectrophotometry (LC-MS) is a technique used to identify and confirm the structures of individual components that have already been separated by HPLC.

The HPLC method works by separating different chemical compounds through their chemical interactions with the stationary and mobile phases in a C_{18} column. Several crucial factors determine a good separation in high performance liquid chromatography; those factors include compound size, charge, and polarity. In addition, the pH of the column is very important. Anthocyanins are most stable as flavyium cations, which require a low pH so it is imperative to select a column that is able to handle acidic pH's. Basically, the primary structure of all anthocyanins are the same, so the only distinct feature that distinguishs them is based on the number and location of both hydroxyl groups and methyl groups, which subsequently differentiate different anthocyanins through different retention times. Reversed-phase C_{18} columns are commonly used for most anthocyanins analysis (Rodrizuez-Saona and Wrolstad, 2001). A reversed-phase C_{18} column consists of nonpolar silica with polar mobile phases (Rodiriguez-Soana and Wrolstad 2001). The wavelength used in the HPLC analysis of anthocyanins is 520nm because the maximum absorbance for most anthocyanins is located between 514-528nm (Mazza and Miniati 1993; Mazza 1997).

The anthocyanins found in purple-fleshed sweetpotato had been identified with HPLC. The results revealed that there is a variation in the anthocyanin composition among the clones (Yoshinaga et al., 1999). All clones had six

distinct anthocyanins designated as YGM-1 through YGM-6. The identity of different anthocyanins detected is determined by comparing them with six standards of purple-fleshed sweetpotato anthocyanins extracted from the sweetpotato cultivar “Yamagawamurasaki (YGM).” The chemical structure of the six YGM pigments is as follows: YGM-1, cyanidin 3-(6,6’-caffeyl*p*-hydroxy-benzoylsophoroside)-5-glucoside and cyanidin 3-(6,6’-dicaffeylsophoroside)-5-glucoiside, YGM-2, cyanidin 3-(6-caffeylsophoroside)-5-glucoside; YGM-3, cyanidin 3-(6,6’-caffeylferulylsophoroside)-5-glucoside, YGM-4, peonidin3-(6,6’-dicaffeylsophoroside)-5-glucoside; YGM-5, peonidin 3-(6,6’-caffeyl*p*-hydroxybenzoylsophoroside)-5-glucoside and peonidin3-(6-caffeylsophoroside)-5-glucoside; YGM-6, peonidin 3-(6,6’-caffeylferulylsophoroside)-5-glucoside.

The HPLC analysis also indicated that there was a range of ratios of peonidin and cyanidin, 0.02 to 24.6 among the sweetpotato cultivars analyzed by Yoshinaga et al. (1999). Therefore, the clones were classified into two major groups based on the peonidin/cyanidin ratio. The results from both HPLC and color analyses indicated that red color predominate for the peonidin type, whereas for the cyanidin type, the blue color predominate. The HPLC analysis was carried out on a Shimazu LC-9A liquid chromatograph equipped with Inertsil ODS-2. Anthocyanins were separated by linear gradient elution for 40 min with 25 to 85% solvent B (1.5% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1.5% H_3PO_4 in H_2O. The temperature of the column was 35 ^{0}C with a flow rate of 1 ml per min. Color value measurement is based on the Hunter colorimetry color reflectance value of L*, a* and b*. The L* value is related to the brightness,

a* and b* values are related to the red-green and yellow-blue color. Samples with a ratio of -1.4 (peonidin/cyanidin) and below are categorized as blue dominant group, whereas samples with a ratio of –1.1 and above are categorized as red dominant group. Therefore, the anthocyanins content and composition can be estimated based on the analytical measurement of colors (Yoshinaga et al., 1999).

Tian et al. (2005) analyzed the anthocyanin compositions of purple-fleshed sweetpotato using HPLC and electrospray ionization tandem mass spectrometry. Selected reaction monitoring (SRM) MS/MS was used to screen and characterize anthocyanins in sweetpotato cell line cultures. The results indicated that there were a total of twenty-six different anthocyanins isolated from the purple-fleshed sweetpotato and all the anthocyanins were either cyanidin or peonidin and their acylated derivatives. In addition, two pelargonidin derivatives, were also identified for the first time.

Rodrizuez-Saona and Wrolstad evaluated the anthocyanins composition of red potatoes (*Solanum buberosum* and *S. stenotomum*) using HPLC. The HPLC separated anthocyanins were also identified by LC-MS. The HPLC results indicated that all red potato samples have similar pigment profiles. The major anthocyanins were 3,5-diglycosides acylated with cinnamic acids. The combined results of both HPLC and MS identified the molecular ions as: MW 741 corresponded to pelargonidin-3-rutinoside-5-glucoside (Pg-3-rut-5-glu), MW 887 and 916 corresponded to pg-3-rut-5-glu acylated with *p*-coumaric acid and ferulic acids, respectively. Anthocyanins acylated *p*-coumaric acid was the major

anthocyanin (70%) in the red-fleshed potatoes (Rodriquez-Saona and Wrolstad, 2001).

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CHAPTER 2

Total Antioxidant Activity, Polyphenols and β-Carotene of Sweetpotato Clones with Varying Flesh Colors

ABSTRACT

Depending on flesh color (white, yellow, orange, purple), sweetpotatoes can be a rich source of carotene, anthocyanins, and other phenolic compounds, which are solubilized in lipophilic or hydrophilic solvents. Most of the reported antioxidant activities in sweetpotatoes have been based on the hydrophilic fraction. In order to obtain the total antioxidant activity (TAA), both hydrophilic and lipophilic fractions need to be considered. The objectives of this study were to determine the hydrophilic and lipophilic antioxidant activity of sweetpotatoes, and calculate their correlations with total phenolic and β-carotene contents in different cultivars of sweetpotatoes. Freeze-dried powders of nineteen sweetpotato cultivars with varying flesh colors were extracted with hexane and acidified methanol. Antioxidant activity (Trolox equivalent, TE μM/g fwb) was measured using three methods: oxygen radical absorbance capacity (ORAC), 2,2 diphenyl-1-picrylhydrazyl (DPPH), and 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid (ABTS). Total phenolics were measured using the Folin-Ciocalteau method, total anthocyanins by the pH-differential method, and β-carotene by HPLC.

The TAA value was highest (27.2 μM TE/g fwb) for NC 415 (purple-fleshed) and lowest (2.31 μM TE/g fwb), for clone no. 13-1 (white-fleshed). The hydrophilic antioxidant activities were highly correlated with the total phenolic contents (R^2=0.938), and the lipophilic antioxidant activities were correlated with the β-carotene contents (R^2=0.863). There were good correlations between ORAC and ABTS (R^2=0.774) as well as ORAC and DPPH (R^2=0.846), indicating

that these methods have similar predictive capacity for sweetpotato antioxidant activities. Purple-fleshed sweetpotatoes have high total antioxidant activity, anthocyanins, and other phenolic compounds. These characteristics suggest that they are a healthy food for consumers, as well as a potential source for natural food colorants.

INTRODUCTION

Fruits and vegetables are a rich source of phytochemicals such as carotneoids, flavonoids and other phenolic compounds. Studies have indicated that these phytochemicals, especially polyphenols, have high antioxidant activity that helps to reduce the risk of cardiovascular disease, cancer, and age-related neuronal degeneration, such as Alzhimer's and Parkinson diseases (Ames et al., 1993). The free radicals are generated in the human body through aerobic respiration and exist in different forms including superoxide, hydroxyl, hydroperoxyl, peroxyl and alkoxyl radicals. Generally, natural antioxidant enzymes in healthy individuals neutralize these free radicals (Rimbach et al., 2005). However, dietary antioxidants are helpful in assisting the body to neutralize free radicals. Therefore, it is important to consume a diet rich in antioxidants such as fruits and vegetable to reduce the harmful effects of oxidative stress (Xiang et al., 2001).

Sweetpotatoes (*Ipomoea batatas L.)* are rich in dietary fiber, minerals, vitamins, and antioxidants such as phenolic acids, anthocyanins, tocopherol and β-carotene (Woolfe, 1992). Besides acting as antioxidants,carotenoids and phenolic compounds are also responsible for giving sweetpotatoes their distinctive flesh colors (cream, deep yellow, orange and purple). Anthocyanins and chlorogenic acid are the major phenolic compounds present in purple colored sweetpotatoes (Suda et al., 2002, Walter and Purcell, 1979). The phenolic contents of the sweetpotatoes cultivated in the US ranged from 117 to 467 mg of chlorogenic acid equivalent/100g of fresh weight (Walter and Purcell,

1979). Purple-fleshed sweetpotatoes have been found to contain up to 60 mg anthocyanins/100g fwb (Suda et al., 2003). In recent years, several reports have indicated that the phytochemicals found in sweetpotato have antioxidant activity and exert several health-promoting functions in humans (Konczak-Islam et al., 2003). Purple-fleshed sweetpotato has been reported to have higher antioxidant activity and phenolic content than a certain variety of blueberry, which as a crop, are reportedly have exceptionally high levels of antioxidants (Cisneros-Zevallos and Cevellos-Casals, 2003). The anthocyanins isolated from purple-fleshed sweetpotatoes were found to have high antioxidant activity (Suda e al., 2002). Cao et al. (1996) included an unknown sweetpotato cultivar in their study and reported that its antioxidant activity was lower than those of leafy vegetables and broccoli. However, it was in the range of other vegetables such as carrot, squash and potato. With the flesh color varying from white, yellow, orange to purple among the sweetpotato cultivars, the antioxidant capacity of sweetpotato roots may vary over a wide range.

Several methods are available to evaluate antioxidant activities of natural compounds in foods or biological systems. Two of the commonly used procedures are the ABTS and DPPH procedures which involved using 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl 1-picrylhydrazyl (DPPH) as free radical generators, respectively. The mechanisms of both methods are similar in that the absorption spectra of the stable free radical changes when the molecule is reduced by an antioxidant or a free radical species. ABTS is soluble in both aqueous and organic solvents and it reacts

relatively fast compared to DPPH which normally takes 1-3 hours for the reaction to be completed. Color interference of DPPH assay with samples that contain anthocyanins leads to underestimation of antioxidant activity. However this problem does not occur with ABTS assay (Arnao, 2000). Nevertheless, the DPPH procedure has been commonly used to assay the antioxidant activity of sweetpotato (Cisneros-Zevallos, 2003; Oki et al., 2002; Rabah et al., 2004)

Oxygen Radical Absorbance Capacity (ORAC) is another method used to measure antioxidant capacity in vitro. This method is based on the inhibition of the peroxyl-radical-induced oxidative initiated by thermal decomposition of azo-compounds such as 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH).The advantage of this method is its ability to react with both hydrophilic and lipophilic antioxidants and to obtain total antioxidant activity. Randomly methylated β-cyclodextrin (RMBC) was used to increase the water solubility of lipophilic antioxidants, which consist of mostly vitamin E related compounds and carotenoids (Huang et al, 2002). Wu et al. (2004) analyzed the antioxidant activity of a sweetpotato cultivar based on the hydrophilic and lipophilic ORAC procedures. They found the total antioxidant activity to be 9.02 μM TE/g fw. The ORAC method is that it uses biologically relevant free radicals, integrates both time and degree of anti-oxidant activity into one data value, and it is readily adaptable to a high-throughput assay system (Prior et al. 2003). The disadvanges is that, this method usually requires the use of a specialized instrument.

Several studies have reported correlations among different antioxidant analysis methods as well as the correlations between those methods and phytochemical concentrations in fruits and vegetables (Akiwa et al., 2003). However, the information on this relation is very limited for sweetpotatoes. It is important to establish a suitable and rapid method to assay the antioxidant capacity of sweetpotato cultivars and processed products that can be used in breeding and food processing research programs. The information on the antioxidant activity of sweetpotatoes would be helpful in increasing the awareness of the consumers regarding the level of beneficial phytochemicals present in this vegetable.

The objectives of this study were 1) to determine the antioxidant activity of sweetpotatoes using ORAC, DPPH and ABTS assays and to assess the correlations among these methods, and 2) to assess the antioxidant activities of the hydrophilic and lipophilic extracts of sweetpotatoes with the concentrations of phenolic compounds, and β-carotene present.

MATERIALS & METHODS

Chemicals

Trolox (2,5,7,8-tetramethychroman-2-carboxylic acid), Folin-Ciocalteau reagent and fluorescein (FL), Folin-Ciocalteau reagent, chlorogenic acid and β-carotene were purchased from Sigma Aldrich (Milwaukee, WI). 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA), randomly methylated β-cyclodextrin (RMCD) from Cyclodextrin Technologies Development Inc. (High Spring, FL). 2,2-diphynyl-1-picrahdrazyl (DPPH), 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and potassium persulfate were obtained from Sigma-Aldrich (St. Louis, MO). All other reagents were of analytical and HPLC grades.

Storage Root Samples

Nineteen sweetpotato cultivars with varying flesh colors (white, yellow, orange, purple) were analyzed. Four of the clones [Beauregard, Hernandez, Covington (NC98-608), Xushu 18], are grown commercially. The other fourteen breeding lines were obtained from the Sweetpotato Breeding Program, North Carolina State University. These breeding lines were identified a breeding program code (NC415) or based on the family and the selection number in a 5 x 5 mating design. The harvested roots were cured and stored at 13-16˚C and 80-90% relative humidity for 2-3 months before samples were taken for analysis. Commercial sweetpotatoes were readily available, and therefore, two replications were taken from two different batches of each cultivar. For the breeding lines, two samples were taken from a limited harvest of each clone.

Preparation of Raw Storage Roots for Analysis

The storage roots were thoroughly washed with tap water, manually peeled and ground into small pieces with a food processor. Samples were taken for moisture determination following a procedure described by Walter and Purcell (1979). The ground samples were then freeze-dried in a Stoppering Trap Freeze Dryer 77560 (Labconco Corporation, Kansas City, MO) for several days at -45^{o}C to -30^{o}C.. Sample preparation, extraction and analysis were carried out in a laboratory with UV-filtered light to minimize light degradation of components.

Extraction of Lipophilic and Hydrophilic Fractions

Freeze dried Samples were ground to fine powder with a cyclotec mill Rose Scientific, (Ontario, Canada). Five grams of freeze-dried powder were vortexed for two minutes in 25 ml hexane, and the mixture was filtered using a Buchner funnel. The hexane extraction was repeated twice, and the combined lipophilic extracts were evaporated at 50^{o}C using a vacuum evaporator until dryness. The residue after hexane extraction was then extracted two times with 25 ml of acidified methanol (7% acetic acid in 80% methanol) to obtain the hydrophilic fraction. The final volume of the hydrophilic fraction was made to exactly 50 mL with acidified methanol.

Measurement of Antioxidant Activity

Hydrophilic and Lipophilic ORAC Assay

For the lipophilic antioxidant assay, the dried hexane extract was re-dissolved in 50 mL of 50% acetone/50% water containing 7% randomly methylated β-cyclodextrin solution, (RMCD) (Huang et al., 2002). The volume of

lipophilic extract was adjusted to exactly 50 mL. Any further dilution was made with the 7% RMCD solution. The 7% RMCD solution was also used as a blank and to dissolve the Trolox standards for the lipophilic assay. For the hydrophilic assay, phosphate buffer (0.075M) was used. The phosphate buffer was also used as a blank and to dissolve the trolox standards for the hydrophilic assay.

The oxygen radical absorbance capacity (ORAC) procedure was carried out following the procedure established by Prior et al. (2003). The fluorescence intensity measurement was performed using a Safire monochromator based microplate reader equipped with a Magellen V4-W reader software (Tecan USA, Research Triangle Park, NC) with the sample loaded on a Costar polystyrene flat-bottom 96-well plate (Corning, Acton, Massachusetts). The concentrations of reagents prepared were identical to those of Prior et al. (2003), except that the samples were diluted 100-fold. The diluted samples, 20 µL, were mixed with 120 µL fluorescein solution in a clear 96-well microplate and incubated at 37 ^{o}C for 15 minutes. Then, 20 µL of AAPH was rapidly added to each well using a multichannel pipet. Immediately following the addition of AAPH, the plate was agitated for 5 s prior to the first reading and for 2 s before each subsequent reading. Readings were done at 1 min intervals for 80 min. Excitation and emission filter wavelengths were set at 484 and 520 nm, respectively. Data were expressed in µM Trolox equivalents (TE) per gram of sweetpotato on a fresh weight basis.

The ORAC values were calculated by using a regression equation ($Y = a + bX$, linear; or $Y = a + bX + cX^2$, quadratic) between concentration (Y) (µM) and

the net area under the fluorescence decay curve (X). Linear regression was used in the range of 6.25-50 μM Trolox. The area under curve was calculated as follow:

$$AUC = (0.5 + f_5/f_4 + f_6/f_4 + f_7/f_4 \ldots\ldots + f_i/f_4) \text{ X } CT$$

where f_4 is the initial fluorescence reading at cycle 4, and f_i is the fluorescence reading at cycle I, and CT is the cycle time in minutes.

The net area under the curve was obtained by subtracting the blank value from that of a sample or standard.

Assay of DPPH Radical Scavenging Activity

Samples were diluted (1:10) with ethanol and the assay was performed following the procedure described by Brand-Williams et al. (1995) with minor modifications.

The diluted sample, 0.1 mL, was pipetted into 3.9mL of DPPH solution to initiate the reaction. The absorbance was read every minute at 515 nm for 180 minutes using the SAFIRE microplate reader equipped with a Magellan V4-W reader software (Tecan USA, Research Triangle Park, NC). The absorbance increase reached a plateau within the 3 hr sampling period which indicated that the reaction had reached completion. Therefore, a reaction time of 3 hours was used for all the DPPH assays. Ethanol (95%) was used as a blank. Trolox (0, 0.1, 0.2, 0.3, 0.4 and 0.5 mM) was used as a standard. Analysis was done in triplicate for each sample and each concentration of standard. The antioxidant activity was reported as micro molar of trolox equivalents per gram fresh weight (μM TE /g fw).

Assay of ABTS Radical Scavenging Activity

ABTS radical scavenging activity activity was determined by a procedure reported by Miller and Rice-Evans (1997). The ABTS solution was prepared by mixing 8 mM of ABTS with 3 mM of potassium persulfate in 25 mL of distilled water. The solution was held at room temperature in the dark for 16 hours before use. The $ABTS^{\cdot+}$ solution was diluted with 95% ethanol (approximately 600 uL ABTS to 40 mL 95% ethanol) in order to obtain an absorbance between 0.8 to 0.9 at 734 nm. Fresh ABTS solution was prepared for each analysis. Antioxidant or standard solutions, 20 uL, were mixed with 1 mL of diluted $ABTS^{\cdot+}$ solution and incubated at 30 ^{0}C. The absorbance at 734 nm was read every minute for 30 min using the SAFIRE microplate reader equipped with a Magellan V4-W reader software (Tecan, Raleigh). Ethanol (95%) was used as a blank.

Trolox with concentrations from 0 to 500 μM were used as a standards. The free radical scavenging activity was expressed as micromolar of trolox per gram of sample (μM TE/g fw).

Measurement of Antioxidant Content

Total Phenolic Assay

Total phenolic compounds were measured using the modified Folin-Ciocalteu method (Singleton et al., 1999). This procedure involved the addition of 0.5 mL Folin-Ciocalteau reagent to samples or standards and let it react for 3 minutes. The samples consisted of the hydrophilic extracts. Each sample or chlorogenic acid standard (0.5 mL) was diluted with distilled water to 5.0 mL. One ml of 1 N sodium carbonate was added to the sample and the mixture was

incubated for reaction for an hour. The absorbance was measured at 725 nm using SAFIRE microplate reader equipped with a Megellan V4-W reader software (Tecan, Raleigh). Distilled water was used as a blank. The content of phenolic compounds was reported as of miligram chlorogenic acid equivalents per 100 gram fresh weight sample (mg CAE/100g fw).

Total Anthocyanin Assay

Total anthocyanin content was measured by the pH-differential method as described by Rodriquez-Saona and Wrolstad (2001). Samples were diluted with two different solutions: potassium chloride (0.025 M), pH 1.0, and sodium acetate (0.4 M), pH 4.5. The pH were adjusted with concentrated hydrochloric acid. Samples were diluted to give an absorbance at 530 nm < 1.2. Water was used as the blank. Diluted samples were held for 15 min before taking the absorbance readings.

The absorbance was measured at wavelength 530 nm and at 700 nm. Difference between the pH 1.0 and pH 4.5 samples was calculated.

$$A = (A_{530nm} - A_{700nm})_{pH\ 1.0} - (A_{530nm} - A_{700nm})_{pH\ 4.5}$$

The monomeric anthocyanin pigment concentration was calculated using the following equation:

Monomeric anthocyanin pigment (mg/liter)

$$= (A \times MW \times DF \times 1000)/ (\varepsilon - l)$$

MW is the molecular weight, DF is the dilution factor, l is the path length, and ε is the molar absorptivity of cyanidin-3-glucoside, which was used as a

standard. The total monomeric anthocyanins were reported on the basis of mg/100g fw sweetpotato.

β-carotene Content

Freeze-dried powder, 5g, was mixed with approximately 2g of calcium carbonate, 1g of diatomaceous earth, and 25ml of methanol. Next, 50ml of a hexane-acetone (1:1) mixture was added and stirred. The mixture was filtered under vacuum through a funnel with a fritted disk. The residue in the funnel was washed two more times with 25ml of methanol and 50ml of the hexane-acetone mixture. All of the extracts were combined in a 250ml separatory funnel and washed with water. A few drops of saturated sodium chloride solution were added to the funnel to facilitate phase separation. The aqueous phase was discarded and the upper layer was transferred to a 50ml volumetric flask and made to volume with hexane (Chandler & Schwartz 1988). Samples were stored in dark vials at -20 ^{o}C until analysis.

The carotene content was analyzed by HPLC. The HPLC system (ThermoQuest San Jose, CA) consisted of a P2000 binary pump, AS 3000 autosampler, and SCM 1000 degasser. Samples were placed in the sample tray cooled to 6 ^{o}C. Aluminum foil was placed over the sample tray to minimize light. Samples, 20 µL, were injected onto a Sunfire C_{18} reverse phase column (4.6 x 100mm, 3.5 µm) (Waters Associates, Milford, MA). Separation was done at 35 ^{o}C with a mobile phase of methanol, acetonitrile and chloroform (42.5/42.5/15 v/v). The flow rate was maintained at 1.2 ml/min. Peaks were monitored at 450nm with a UV 6000 LP Diode Array Detector. Standard solutions of β-carotene with

concentrations from 0.5 µg/ml to 10 µg/ml. Thermoseperations were used to obtain a standard curve. ThermoQuest Chromatography Data Acquisition Software version 4.1 was used to collect and process the data.

Statistical Analysis

Group differences were evaluated using t-tests with $p<0.05$ considered to be a statistically significant difference. Means were compared with Duncan's multiple range test with $\alpha = 0.05$ using the SAS Statistical Analysis System v8.1 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Oxygen Radical Absorbance Capacity (ORAC)

The typical curves of the ORAC-fluorescence intensity over time for the hydrophilic or lipophilic fraction of several sweetpotato breeding lines are shown in Figure 1. NC415 exhibited the largest area under the curve corresponding to high antioxidant activity, followed by Beauregard, Covington and Xushu 18 cultivars.

Figure 2 shows the total antioxidant activity of 19 sweetpotato clones. The total antioxidant activity of the white and yellow fleshed clones (Xushu 18, 11-12, 13-10) were lowest (0.19-1.80 μM TE/g fwb) most of the orange fleshed samples were in a middle range of 5.14 to 10.27 μM TE/g fwb, except the 11-20 clone, a dark orange flesh sample with 18.40 μM TE/g fwb. The total ORAC antioxidant activity of 9.02 μM TE/g fwb for a sweetpotato clone was reported by Wu et al. (2004) is within this range. On the other hand, the dark purple-fleshed clones (NC 415, 12-5, 12-9, 13-17, 13-18) were in the upper range of 8.50 – 27.20 μM TE/g fwb, with NC415 having highest ORAC.

The results indicated that the antioxidant activity varied widely among the sweetpotato clones. The color intensity of purple-fleshed sweetpotatoes tended to be associated with high total antioxidant activity. Aside from sweetpotatoes, other food commodities, such as sorghum, also show a similar relationship between the color intensity and antioxidant activity. Brown and black sorghums had the highest levels of freely extractable polyphenols. The total antioxidant activity of these sorghum clones was in the range of 271-878 μM TE/g dwb as

compared to less than 25 μM TE/g for a white clone (Akiwa et al., 2003). Bao et al. (2005) reported a similar trend for Chinese bayberries in which the black bayberry cultivars had much higher antioxidant activity than those the pink and yellowish varieties.

The constituents responsible for the hydrophilic antioxidant activity are primarily phenolic compounds and anthocyanins, whereas carotenoids and tocopherol are the main constituents in lipophilic extracts. The hydrophilic and lipophilic antioxidant activities ranged from 1.28 to 25.7 μM TE/g fwb and 0.249 to 2.76 μM TE/g fwb respectively. Wu et al. (2004) reported the hydrophilic and lipophilic antioxidant activity of a single sweetpotato cultivar to be 8.58 and 0.44 uM TE/g fwb. NC 415 (purple-fleshed) had the highest hydrophilic antioxidant activity, while clone 11-20 (orange fleshed) had the highest lipophilic antioxidant activity (Fig. 2). The differences between hydrophilic and lipophilic antioxidant activity were significant for all cultivars of sweetpotatoes ($P<0.05$). β-carotene also provided the distinctive orange color of the root in orange sweetpotatoes. Similarly, carrots, which derive their color from β-carotene, also have high lipophilic antioxidant activity (Wu et al., 2004). Blueberry was among the fruits with high hydrophilic antioxidant activity, 61.8 μM TE/g fwb, and avocado was among the vegetables with high lipophilic antioxidant activity, 5.52 μM TE/g fwb (Wu et al, 2004).

For all samples, the hydrophilic antioxidant activity accounted for more than 85% of the total antioxidant activity. These results are in accordance with other food commodities reported by Wu et al. (2005), where the hydrophilic

antioxidant activity also accounted for approximately 85% of the total antioxidant activity. Kurilich et al. (2002) analyzed the antioxidant activity of broccoli using the ORAC procedure. The results also indicated that there is a wide range of antioxidant activity among different cultivars of broccoli. The hydrophilic extract contained 80-95% of the total antioxidant activity of broccoli. Kurilich also suggested that lipophilic extracts produced very little antioxidant activity because the ORAC assay is an aqueous system. So, it may not accurately measure the antioxidant activity of non-polar compounds. Prior et al. (2005) suggested that the radical used in ORAC procedure might not be suitable for lipophilic antioxidants such as β-carotene.

There are advantages and disadvantages of the ORAC procedure. One of the major advantages for adapting ORAC procedure is that the assay of this analysis involves a controllable source of peroxyl radicals that mimic the reactions of antioxidants with lipids in both food and physiological systems, therefore it can be used to detect both hydrophilic and lipophilic antioxidants by altering the radical source and solvent (Ou et al., 2001). However, some researchers suggested that the ORAC procedure is carried out with the assumption that the antioxidant mechanism and protection of fluorescein by antioxidants mimics critical biological substrates. Therefore, if the reaction mechanism between antioxidant and fluorescein does not actually reflect the real reaction in biological substrates, then this procedure may not reflect the real antioxidant capacity of samples (Frankel and Meyer, 2000).

DPPH Radical Scavenging Activity

The sample with the highest antioxidant activity was NC-415, 1028 μM TE/g fw, which was a dark purple-fleshed sweetpotato. White-fleshed sweetpotato clone 11-12 had the lowest antioxidant activity with a value of 13.0 (M TE/g fw, and Xushu-18 72.0 μM TE/g fw (Figure 3). Samples with DPPH values between these extremes were either orange or yellow fleshes. Statistical analysis indicated that Beauregard, Hernandez, Covington, and clone12-9 were not significantly different ($P>0.05$) among one another. In addition, clones 11-5, 11-16, 12-7, 13-14 and 13-15 were not significantly different in DPPH values.

Kano et al. (2005) analyzed the antioxidant activity of purple-fleshed sweetpotato and other fruits and vegetables such as red cabbage, grape and purple corn using on the DPPH assay. The results indicated that anthocyanins from purple-fleshed sweetpotato had a highest DPPH radical-scavenging activity compare to other fruits and vegetables evaluated. Similarly, Rabah et al. (2004) found that the extract from baked sweetpotato had strong radical scavenging activity based on DPPH analysis. The extracts of sweetpotatoes were fractionated to four fractions. Two fractions with high DPPH radical scavenging activity also had high concentrations of phenolic compounds. Analysis on colored sorghum cultivars also showed a wide range of antioxidant activity (Awika et al., 2003).

DPPH is generally a more cost efficient method to analyze antioxidant activity. The test is simple and needs only a UV-vis spectrophotometer to

perform. Prior found that good reproducibility with the DPPH assay (Prior et al., 2005).

ABTS Radical Scavenging Activity

ABTS radical scavenging activity is shown in Figure 4. The sample with the highest antioxidant activity was NC-415, 1617μM TE/g fwb, while clone 13-1 had the lowest antioxidant activity, 805 μM TE/g fwb. The clones 11-20, 12-5, 12-9, 13-17 and 13-18 were not significantly different ($P \geq 0.05$) in ABTS values. Beauregard, Hernandez, Covington, Clones 11-5, 12-7, 13-6 and 13-14 were also not significantly different ($P \geq 0.05$). These results are essentially the same as those obtained with the ORAC and DPPH methods.

Both ABTS and DPPH procedures are relatively fast and easy methods to perform. However, the ABTS method offers a number of advantages over the DPPH assay. The ABTS method can be used in a wide pH range (Cano et al., 1998), whereas DPPH method is limited to higher pH applications. In addition, since ABTS is soluble in aqueous and organic solvents, the assay has been used to measure hydrophilic and lipophilic antioxidants by doing the assay in buffered media and organic solvents (Cano et al., 2002), or by partitioning antioxidant in mixtures between hexane and aqueous solvents (Wu et al., 2004). However, water-soluble reactions appear to be favored (Pulido et al., 2003). Additionally, the reaction of ABTS is much faster than DPPH assay. Thermodynamically, antioxidant compounds can only react with ABTS if they have a redox potential lower than that of ABTS (0.68 V). Most phenolic compounds have lower redox potentials than ABTS. Furthermore, unlike the DPPH method, color interference

is not an issue for ABTS. Color interference of DPPH with samples that contain anthocyanins leads to underestimation of antioxidant activity.

Total Phenolic Content

The total phenolic content was the highest, 79.2 mg CAE/100g fwb, for NC- 415 (purple-fleshed) and lowest, 0.3 mg CAE/100g fwb for Xushu 18 (white-fleshed) (Table 1). In general, purple-fleshed sweetpotato clones had the highest total phenolic content followed by orange, yellow and white-fleshed cloness, respectively. The orange-fleshed clone, 11-20 which had high antioxidant activities (Figure 2, 3, 4) also contained substantial amounts of phenolic compounds (47.8mg/100g fwb). Walter and Purcell (1979) also reported that the total phenolic contents varied widely among sweetpotato clones with the highest amount 75 mg CAE/100g fwb. The phenolic contents in sweetpotato storage roots were much lower than the values of 1.42-17.2 g/100g dw observed in sweetpotato leaves (Islam et al., 2002). Aside from genetic factors, the growing conditions may play an important role in the formation of secondary metabolites, including phenolic acids (Fernando Reyes and Cisneros-Zevallos, 2003; Wu et al., 2004).

Among all fruits, berries represent one of the most important sources of phenolic compounds. Amakura et al. (2002) reported that among all berries that they analyzed, black current had one of the highest phenolic contents resulting in high radical scavenging activity. The average phenolic content of black current samples was 2,100 mg CAE/100g fwb. The total phenolic content of blueberries and strawberries ranged from 430 to 1990 mg gallic acid/ kg fw (Ehlenfeldt and

Prior, 2001) and 16,000 to 24,100 mg gallic acid/kg fw (Kahkonen et al., 2001) respectively.

Total Anthocyanin Content

There was a wide variation observed in the total anthocyanins content among the sweetpotato breeding lines. The total anthocyanins content of the samples ranged from 1.32 to 113 mg/ 100 g sample fwb (Table 1). Among all the samples, nine of the sweetpotato clones with purple and orange flesh had detectable anthocyanins content. The 11-20 clone with high antioxidant activity (18.2 mg CAE/g fwb) had undetectable anthocyanin content. High anthocyanin contents with peonidin and cyanidin as the major components in purple and red fleshed sweetpotatoes have been reported by several investigators (Furuta et al., 1998; Harada et al., 2004; and Yoshinaga et al., 1999). Cisneros-Zevallos and Cevallos-Casals (2003) reported that the anthocyanin content of red-fleshed sweetpotato was 182 mg anthocyanin/100g fw.

For a comparison with sweetpotato, red-fleshed potato (*Solanum tuberosum*) was also reported to contain anthocyanin pigments. Rodriguez-Saona and Wrolstad (1998) reported anthocyanin contents ranging from 2 to 40 mg/100g fwb for 33 different cultivars of red-fleshed potato. Blueberries have been reported to have one of the highest anthocyanins content among all fruits and vegetables. The total anthocyanin content of different cultivars of blackberries ranged from 116.6 to 152.8 mg anthocyanins /100g fwb (Sellappan et al., 2002, Wang and Lin, 2000). The anthocyanins content also varied among different cultivars of blackcurrants, ranging from 156 to 1,064 mg

anthocyanins/100g fw (Iversen, 1999). Howard et al. (2003) analyzed blueberries from five commercial cultivars and 13 breeding lines for the phenolic content and total anthocyanins. They reported that the anthocaynin content of blueberries was as high as 249mg/100g fwb. Based on that, the anthocyanin content of purple-fleshed sweetpotato would rank in the middle among fruits and vegetables that have been analyzed.

β-carotene Content

Orange-fleshed sweetpotato had the highest β-carotene content among the cultivars of sweetpotato. Hernandez and clone 11-20, which are both dark orange-colored, had the highest β-carotene content with 166 and 228 μg/g fwb, respectively (Table 1). Therefore, the high antioxidant activity of clone 11-20 can be attributed to its phenolic compound and β-carotene contents since anthocyanins were not detectable. On the other hand, the white-fleshed sweetpotato, Xushu 18, had the lowest β-carotene content, 0.19 μg/g fwb. Kays and his group (Simonne et al., 1993) analyzed the β-carotene content in various sweetpotato breeding lines and also observed a wide range of β-carotene contents, ranging from 1 to 190 μg/g dwb.

The β-carotene content varies widely with fruits and vegetables. The reported β-carotene content of broccoli, carrot, clementine, nectarine, tomato and Victoria plum were 3590, 46180, 950, 570, 13560 and 1010 μg/g fwb respectively (Roberts and Gordon, 2003). The results suggested that fruits and vegetables with intense orange and red colors normally have higher β-carotene levels.

Correlations

There were good correlations among the antioxidant activities measured by ORAC, ABTS and DPPH suggesting that these methods have similar predictive capacity for sweetpotato antioxidant activities. The R^2-values between the ORAC and DPPH procedures, and between ORAC and ABTS procedures were 0.846 (Fig. 5) and 0.774 (Fig. 6) respectively. The correlation between the ABTS procedure and the DPPH procedure were also highly significant (R^2 = 0.888) (Fig. 7). Analysis of antioxidant activity in other food commodities, such as sorghum, cocoa, green tea, red wine showed similar correlations between the three procedures. The R^2-value between ORAC and ABTS procedures, and between ORAC and DPPH procedures were 0.99 and 0.98, respectively, for the antioxidant capacity of sorghum (Awika et al., 2003).

The hydrophilic antioxidant activity data obtained from ORAC procedure were highly correlated with the total phenolic content (R^2=0.938) (Fig. 8). The DPPH and ABTS procedures also correlated well with total phenolic content (R^2=0.870 and R^2=0.863 respectively). Furuta et al. (1998) tested the radical scavenging activity of sweetpotato cultivars with varying fleshed color. The results also showed that purple-flesh sweetpotato had a higher radical scavenging activity. The radical scavenging activity also increased as the content of phenolic compounds increased. The phenolic compounds in other fruits and vegetables showed good correlations with the ORAC hydrophilic antioxidant activity. Stintzing et al. (2005) studied the phenolic contents and hydrophilic antioxidant activity of cactus pear and found correlations of r=0.995 and r=0.985

between the concentration of phenolic compounds and the hydrophilic antioxidants of the juice and the pulp. The phenolic content of other fruits and vegetables such as cowpeas, spinach, blackberry and blueberries also correlated very well with the antioxidant activity measured by the ORAC procedure (Prior et al., 2005).

The antioxidant activity of the lipophilic fraction was highly correlated with the β-carotene content, R^2=0.863 (Fig. 9). Kurilich et al., (2002) analyzed the antioxidant activity of broccoli using the ORAC procedure. The results indicated that the carotenoid content of broccoli was also correlated with the lipophilic antioxidant activity.

In general, among the samples evaluated, dark purple-fleshed sweetpotatoes had high phenolic and anthocyanin contents and antioxidant activities assayed by ORAC, DPPH and ABTS procedures. However, an exception was clone 11-20. This clone with high antioxidant activities (Figure 2 ,3, 4) had the highest β-carotene content (228.9 µg/g fw), an intermediate level of phenolics (47.8 mg CAE/100g fw) but its anthocyanin content was undetectable. This result implied that the phytochemicals other than anthocyanins may have a major contribution to the antioxidant capacity of sweetpotatoes. Based on the limited purple cultivars available, a correlation between the ORAC hydrophilic fraction and anthocyanin content of r=0.84 was observed.

Conclusions

There were good correlations among the antioxidant activity measured by ORAC, ABTS and DPPH. These methods have similar predictive capacity for

sweetpotato antioxidant activities. However, there were pro and cons for each of those analytical methods.

There were also good correlations between hydrophilic antioxidant activities and total phenolic content, and between lipophilic antioxidant activities and β-carotene contents. This indicated that phenolic compounds and β-carotene were responsible for the antioxidant activity in the hydrophilic and lipophilic fractions respectively.

Purple-fleshed sweetpotatoes had the highest levels of total antioxidant activity, phenolic compounds and total anthocyanin content. On the other hand orange-fleshed sweetpotato had the highest level of β-carotene content.

Future Studies

The over all results indicated that among sweetpotato clones with varying fleshed colors, purple-fleshed sweetpotatoes have the highest level of antioxidant capacity as well as bioactive compounds. However, the results were only based on limited number of purple-fleshed sweetpotato clones. Whether all purple-fleshed sweetpotatoes of different variety would also demonstrate higher level of antioxidant and bioactive compound remains unknown. Therefore, future studies should focus of purple-fleshed sweetpotatoes of different breeding lines.

There was a good correlation among different antioxidant analysis procedures and between the antioxidant analysis procedure with the phenolic and β-carotene content. Therefore, future studies can be simplified by studying either the antioxidant activity or the bioactive compounds of the storage roots in order to determine the antioxidant capacity.

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Table 1: Phenolic, anthocyanins and β-carotene content of sweetpotato clones

Sample	Flesh color	Dry Matter, %	Phenols, mg CAE/100g fw	Total anthocyanin, mg/100g fw	β-carotene, ug/g fw
Beauregard	orange	28.4	21.1 ^{f}	ND	92.08 ^{c}
NC 415	purple	26.8	79.2 ^{cb}	113.09 ^{a}	6.28 ^{h}
Hernandez	orange	28.8	15.7 ^{g}	ND	166.32 ^{b}
Covington	orange	27.3	18.3 ^{f}	3.78 ^{e}	120.00 ^{g}
Xushu 18	white	31.1	0.300 ^{k}	ND	0.19 ^{j}
11--5	orange	34.9	12.0 ^{gh}	1.23 ^{f}	55.20 ^{d}
11--12	yellow	28.4	1.0 ^{k}	ND	1.30 ^{i}
11--16	light orange	31.8	9.30 ^{ij}	ND	10.23 ^{g}
11--20	orange	24.7	47.8 ^{c}	ND	228.90 ^{a}
12--5	purple	31.3	38.1 ^{d}	19.61 ^{d}	37.43 ^{e}
12--7	light orange	33.1	9.8 ^{hij}	ND	22.50 ^{ef}
12--9	light purple	32.6	19.0 ^{f}	2.33 ^{f}	17.13 ^{fg}
12--17	light orange	33.5	8.1 ^{j}	ND	8.83 ^{g}
13--1	yellow	32.6	2.6 ^{k}	ND	1.80 ^{i}
13--6	purple	29.7	21.6 ^{e}	5.81 ^{e}	47.65 ^{d}
13--14	orange	29.5	11.0 ^{hi}	ND	38.10 ^{e}
13--15	orange	29.1	12.1 ^{gh}	ND	108.78 ^{b}
13--17	purple	27.6	51.7 ^{b}	29.18 ^{c}	28.38 ^{e}
13--18	purple	33.1	71.6 ^{a}	40.14 ^{b}	4.05 ^{hi}

$^{a-h}$ Means within a column with the same superscript letter are not significantly different (*P*=0.05).
ND means non-detectable.

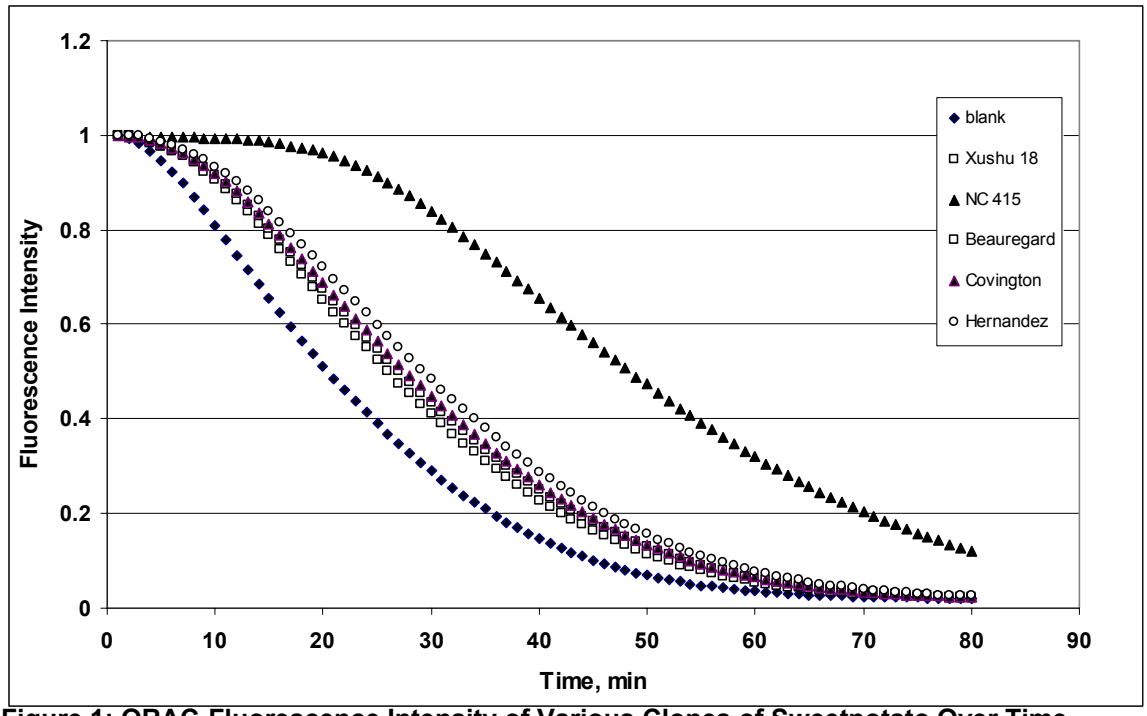

Figure 1: ORAC-Fluorescence Intensity of Various Clones of Sweetpotato Over Time (Hydrophilic Extract)

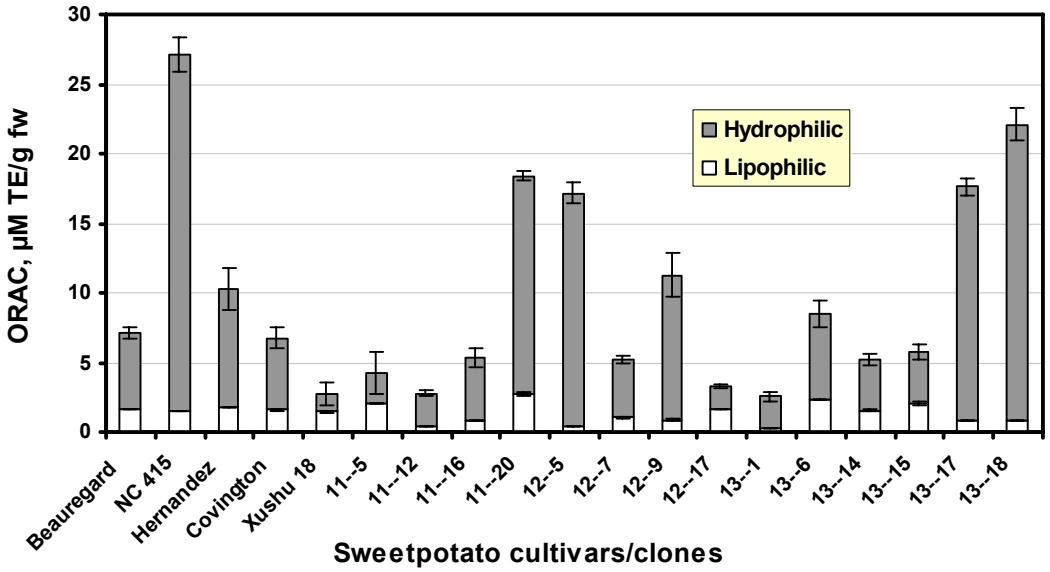

Figure 2: Total Antioxidant Activity of Sweetpotato Clones
Bars indicate standard errors for commercial clones and standard deviation for breeding lines.

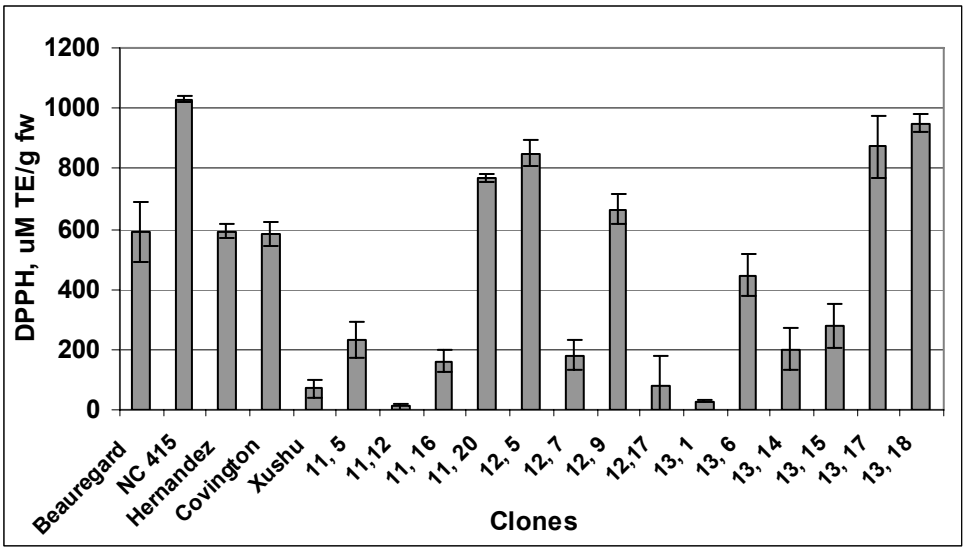

Figure 3: DPPH: Antioxidant Activity of Sweetpotato Clones with Varying Flesh Colors. Bars indicate standard errors for commercial clones and standard deviation for breeding lines.

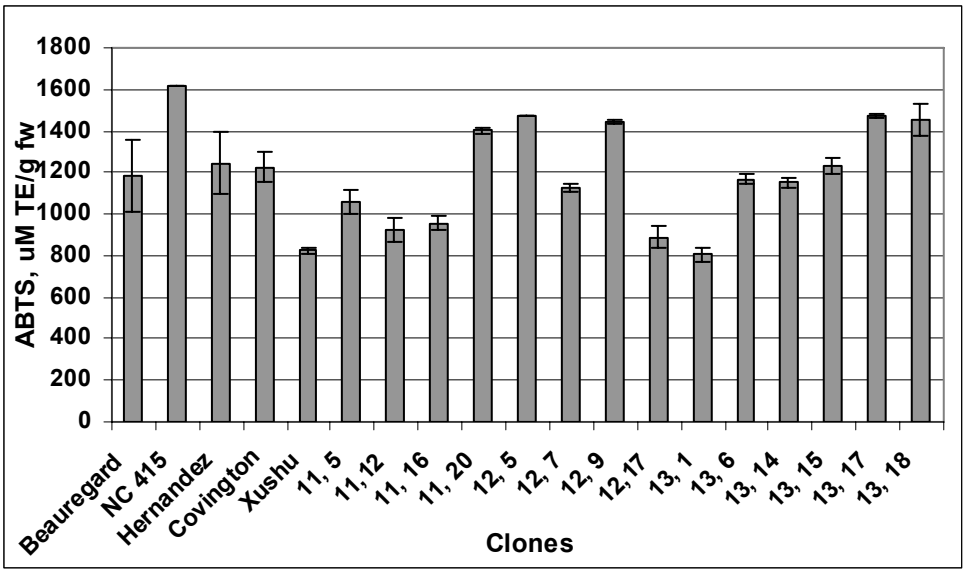

Figure 4: ABTS: Antioxidant Activity of Sweetpotato Clones with Varying Flesh Colors Bars indicate standard errors for commercial clones and standard deviation for breeding lines.

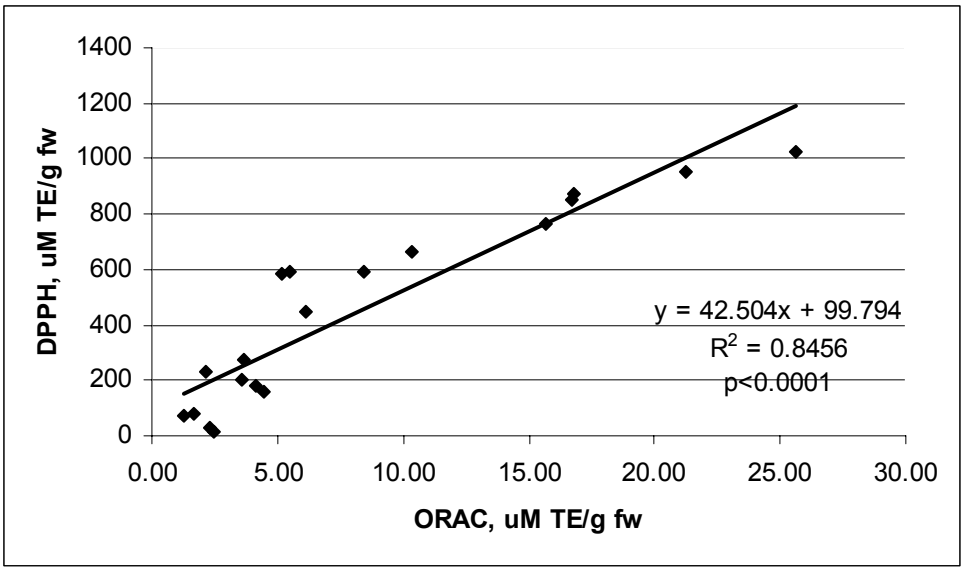

Figure 5: Correlation between ORAC and DPPH

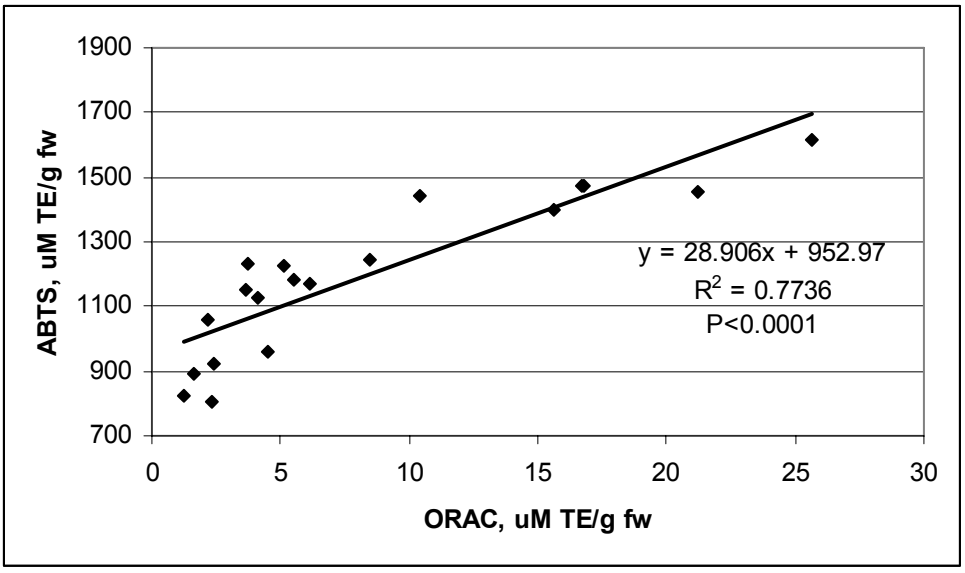

Figure 6: Correlation between ORAC and ABTS

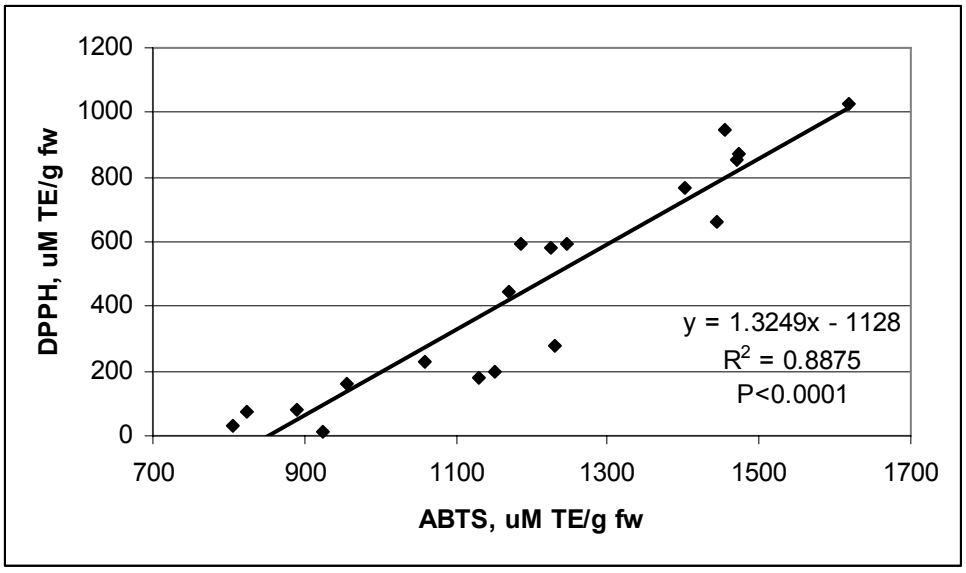

Figure 7: Correlation between ABTS and DPPH

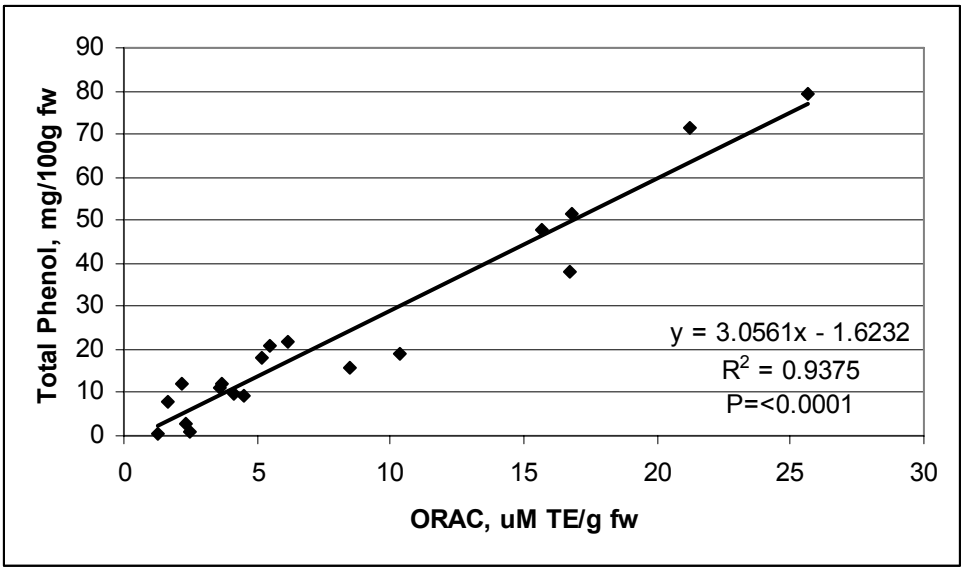

Figure 8: Correlation between ORAC (hydrophilic fraction) and Total Phenols

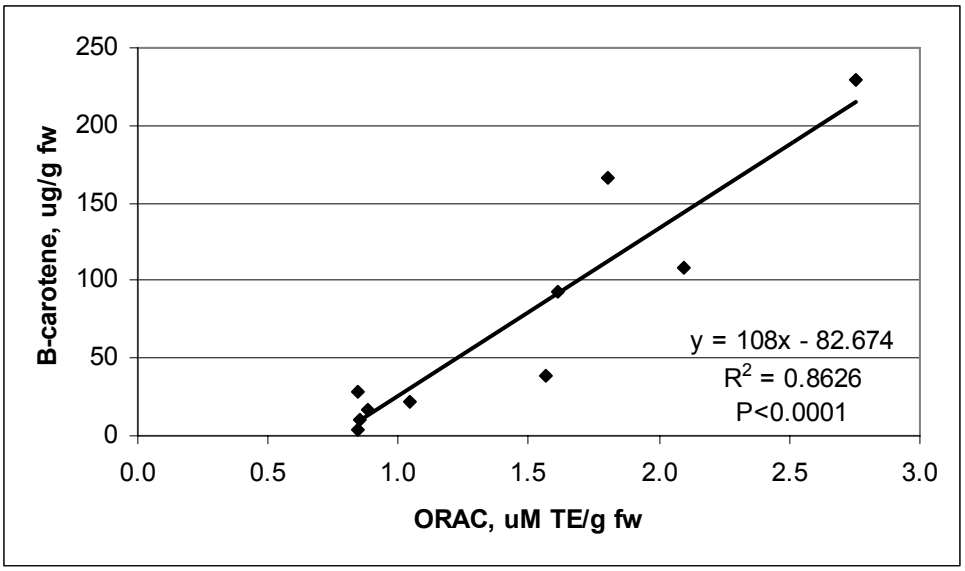

Figure 9: Correlation between ORAC (lipophilic fraction) and β-Carotene Content

CHAPTER 3

Antioxidant Activity and Anthocyanin Characterization of Purple-Fleshed Sweetpotatoes

ABSTRACT

The previous studies have showed that dark purple-fleshed sweetpotatoes have the highest antioxidant activity among the clones with varying flesh colors. Natural food colorants and various processed products derived from high anthocyanin sweetpotatoes are being commercialized in the health food market. Characterization of the pigments in the purple-fleshed cultivars for physiological functionality and polyphenolic compounds in relation to the color of the derived products is important for breeding efforts and for the processing industry. This study aimed to analyze the anthocyanin content and composition of diverse groups of purple-fleshed sweetpotatoes collected from several sweetpotato growing countries. Freeze-dried powders of raw and steamed roots were analyzed for total anthocyanins by the pH-differential method, total phenolic content by Folin-Ciocalteu procedure, and antioxidant activity by (ORAC) analysis. The color values and the anthocyanin composition of the roots were analyzed by colorimetry and HPLC. The results showed that the anthocyanin and phenolic compounds concentrations as well as antioxidant activities varied over a wide range in the purple-fleshed sweetpotato collections. The amount of phenolic compound extracted increased after heat treatment of the sweetpotato samples. This resulted in higher levels of free radical scavenging activity than in extracts from non-heated storage roots. Based on color measurements, the clones evaluated were categorized as a red-dominant group (peonidin-rich). However, HPLC analysis revealed that the ratios of peonidin and cyanidin varied widely

among the samples and that some clones contained more cyanidin anthocyanins than peonidin anthocyanins.

INTRODUCTION

The previous studies have demonstrated that purple-fleshed sweetpotatoes had higher antioxidant activity and phenolic compound concentrations than other sweetpotato types. Recently, purple-fleshed sweetpotatoes have been promoted as a health food in several countries in Asia, especially in Japan. Various processed products derived from high anthocyanin sweetpotatoes are being commercialized with good market potential. These products include natural purple food colorants, purple paste and flour for use in juices, alcoholic beverages, bread, noodles, soup, jam and confectionary (Suda et al., 2003). During the past few years several new sweetpotato cultivars with deep and purple flesh color have been developed in Japan to meet a growing demand in the fresh markets and in the processing industry.

With growing consumer demand for health foods in the United States, purple-fleshed sweetpotatoes may provide a significant market opportunity. However, commercial production of purple-fleshed sweetpotatoes is almost non-existent in this country. Therefore, there is a need to develop the purple-fleshed sweetpotato clones adapt to local growing conditions with high yield and good quality for the fresh consumption and for processing applications, collection and analysis of diverse purple-fleshed sweetpotato germplasm is a necessary step in the development of a breeding program. The characterization of the purple pigments in terms of physiological functionality, anthocyanin content and composition in relation to the color of the derived products from these collections

of purple cultivars is also important for sweetpotato breeders in selecting the best parental materials for hybridization.

Purple-fleshed sweetpotatoes are rich in anthocyanins, which are categorized as either peonidin and cyanidin. The chemical difference between peonidin and cyanidin is the presence or absence of a methoxyl group at the 3'-position of the aromatic B-ring. Theses pigments exist in either mono- or di-acylated forms (Goda, 1997). Among the eight major anthocyanins in the purple-fleshed sweetpotatoes, two of them are mono-acylated by caffeic acid, and the others are di-acylated by caffeic acid alone, caffeic acid and p-hydroxybenzoic acid, or caffeic acid and ferulic acid. One common characteristic among all the types of anthocyanins in purple-fleshed sweetpotatoes is that they are bound to at least one caffeoyl group, which makes them a good free radical scavenger.

Recent research on the nutraceutical properties of purple-fleshed sweetpotatoes indicated that the anthocyanins extracted exhibited strong radical scavenging activity (Oki et al., 2002) and anti-mutagenic activity in a salmonella typhimurium assay (Oki et al., 2002; Yoshimoto et. al., 1999) and significantly reduced high blood pressure and carbon tetrachloride-induced liver injury in rat (Suda, et. al., 1997). Other physiological functions of anthocyanins include anti-inflammatory, antimicrobial, and antihypertensive activities and ultraviolet protection effects (Yoshimoto et al., 2001).

Anthocyanin composition can be determined based on color value measurement and HPLC analysis. Color analysis is based on the Hunter colorimetry color reflectance values of L*, a* and b*. The b*/a* ratio of -1.4 and

below are categorized as blue dominant group with high cyanidin content, whereas samples with a ratio of –1.1 and above are categorized as a red dominant group which is predominantly peonidin. HPLC analysis indicated that there was a range of peonidin/ cyanidin ratio from 0.02 to 24.6 among different purple-fleshed sweetpotato cultivars (Yoshinaga et al., 1999).

The objective of this study was to characterize the anthocyanin content and composition of a diverse group of purple-fleshed sweetpotatoes collected from several sweetpotato growing countries. The total phenolic content, antioxidant activity and color characteristics of this collection were determined. The effect on thermal process of these components was also investigated.

MATERIALS AND METHODS

Chemicals

Trolox (2,5,7,8-tetramethychroman-2-carboxylic acid), Folin-Ciocalteau reagent, chlorogenic acid and fluorescein (FL) were obtained from Sigma-Aldrich (Milwaukee, WI). 2,2'-azobis (2-amidino-propane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA). All other chemicals were of analytical or HPLC grade.

Root Samples

Twenty-one purple-fleshed sweetpotato breeding clones with different shades of purple color were evaluated. This diverse group of purple-fleshed sweetpotatoes was obtained from a collection maintained and developed by the Sweetpotato Breeding Program at North Carolina State University. The germplasm used to make the crosses was obtained from different countries including Puerto Rico, Peru, Japan, The Philippines and Korea. Only limited amount of samples were available. Therefore, instead of replications two repeated measurements were carried out for each clone.

Sample Preparation for Raw and Steamed Storage Roots

The roots were thoroughly washed with tap water, manually peeled, cut longitudinally into halves. One half was steamed for 30 minutes, manually mashed, while the other half was grounded into small pieces with a food processor. The moisture content of raw and steamed samples was obtained by leaving the samples in the oven for 3 hours at 70 ^{o}C and then 17 hours at 105 ^{o}C as described by Walter and Purcell (1979). Both raw and steamed samples were

then freeze-dried in a Stoppering Trap Freeze Dryer 77560 (Labconco Corporation, Kansas City, MO) for several days at -45^{o}C to -30^{o}C. The freeze dried samples were powdered in a cyclotec mill with 1mm mesh (UDY Corp., Fort Collins, CO), and stored at -20C until ready for analysis.

Preparation of Sweetpotato Extracts

The extraction was carried out using a Dionex accelerated solvent extractor (ASE 200) equipped with a solvent controller (Dionex Corp., Sunnyvale, CA). Sweetpotato powder (1g) was mixed with 5 g sea sand, and loaded into a 22 ml extraction cell with cellulose filter paper at the bottom and tightly sealed. The selected variables for the extraction were: pressure, 1500 psi; temperature, 100 ^{o}C; extraction time, 3 x 5 min cycles; flushing volume, 60%; and nitrogen purge time, 60 s. The extracting solvent (7% acetic acid in 80% methanol) was purged with nitrogen gas for 2 hrs prior to use to prevent phenolic oxidation during extraction. The parameters were 5 min static, flush, 60%, purge, 60s, cycle, 3 temperature, 100 ^{o}C (7 % acetic acid in 80% methanol) pressure, 1500 psi. The extracts were collected in UV-proof glass tubes, adjusted to 50ml with the same solvent, and stored at -80 ^{o}C until analyzed.

Measurement of Antioxidant Content

Total Phenolic Assay

Total phenolic compounds were measured using the modified Folin-Ciocalteu method (Singleton et al., 1999). This procedure involved the addition of 0.5 mL Folin-Ciocalteau reagent to samples or standards and let it react for 3 minutes. The samples consisted of the hydrophilic extracts. Each sample or

chlorogenic acid standard (0.5 mL) was diluted with distilled water to 5.0 mL. One ml of 1 N sodium carbonate was added to the sample and the mixture was incubated for reaction for an hour. The absorbance was measured at 725 nm using SAFIRE microplate reader equipped with a Megellan V4-W reader software (Tecan, Raleigh). Distilled water was used as a blank. The content of phenolic compounds was reported as of mg chlorogenic acid equivalents per 100 gram fresh weight sample (mg CAE/100g fw).

Total Anthocyanin Assay

Total anthocyanin content was measured by the pH-differential method as described by Rodriquez-Saona and Wrolstad (2001). Samples were diluted with two different solutions: potassium chloride (0.025 M), pH 1.0, and sodium acetate (0.4 M), pH 4.5. The pH was adjusted with concentrated hydrochloric acid. Samples were diluted to give an absorbance at 530 nm < 1.2. Water was used as the blank. Diluted samples were held for 15 min before taking the absorbance readings.

The absorbance was measured at wavelength 530 nm and at 700 nm. Difference between the pH 1.0 and pH 4.5 samples was calculated.

$$A = (A_{530nm} - A_{700nm})_{pH\ 1.0} - (A_{530nm} - A_{700nm})_{pH\ 4.5}$$

The monomeric anthocyanin pigment concentration was calculated using the following equation:

Monomeric anthocyanin pigment (mg/liter)

$$= (A \times MW \times DF \times 1000)/ (\varepsilon - l)$$

MW is the molecular weight, DF is the dilution factor, l is the path length, and ε is the molar absorptivity of cyanidin-3-glucoside, which was used as a standard. The total monomeric anthocyanins were reported on the basis of mg/100g fw sweetpotato.

Measurement of Antioxidant Activity

Oxygen Radical Absorbance Capacity (ORAC)

The oxygen radical absorbance capacity (ORAC) procedure was carried out following on the procedure established by Prior et al. (2003). The fluorescence intensity measurement was performed using a Safire monochromator based microplate reader equipped with a Magellen V4-W reader software (Tecan USA, Research Triangle Park, NC) with the sample loaded on a Costar polystyrene flat-bottom 96-well plate (Corning, Acton, Massachusetts). The concentrations of reagents prepared were identical to those of Prior et al. (2003), except that the samples were diluted 100-fold. The diluted samples, 20 µL, were mixed with 120 µL fluorescein solution in a clear 96-well microplate and incubated at 37 ^{o}C for 15 minutes. Then, 20 µL of AAPH was rapidly added to each well using a multichannel pipet. Immediately following the addition of AAPH, the plate was agitated for 5 s prior to the first reading and for 2 s before each subsequent reading. Readings were done at 1 min intervals for 80 min. Excitation and emission filter wavelengths were set at 484 and 520 nm, respectively. Data were expressed in µM Trolox equivalents (TE) per gram of sweetpotato on a fresh weight basis.

The ORAC values were calculated by using a regression equation (Y = a + bX, linear; or Y = a + bX + cX^2 , quadratic) between concentration (Y) (μM) and the net area under the fluorescence decay curve (X). Linear regression was used in the range of 6.25-50 μM Trolox. The area under curve was calculated as follow:

$$AUC = (0.5 + f_5/f_4 + f_6/f_4 + f_7/f_4 \ldots\ldots + f_i/f_4) \text{ X CT}$$

where f_4 is the initial fluorescence reading at cycle 4, and f_i is the fluorescence reading at cycle I, and CT is the cycle time in minutes.

The net area under the curve was obtained by subtracting the blank value from that of a sample or standard.

pH Determination

Five grams of sweetpotato paste were dispersed in 10 ml of distilled water at 25 ^{o}C for pH measurement using a Corning pH meter (Corning, Acton, M.A.).

Measurement of Visual Color

The reflectance color values (L*, a*, and b*) of sweetpotato paste and powder samples were measured with a Hunter colorimeter (D25/DP9000 Tristimulus Colorimeter, Hunter Associate Laboratories Inc., Reston, VA). The L* value signifies the lightness (100 for white and 0 for black), a* value represents greenness and redness (-a* for green and + a* for redness), and b* value indicates change from blueness (-b*) to yellowness (+b*). The instrument (45°/0°geometry, D25 optical sensor) was calibrated against a standard white standard tile (L* = 92.75, a* = - 0.76, b* = -0.07). Samples were filled into a 35 mm Petri dish, covered, and carefully pressed against the surface to remove air

bubbles. Measurements were taken at 3 different locations for each specimen, and duplicate specimens were performed for each clone. The average of 6 readings per clone is reported.

HPLC Analysis of Anthocyanins Extracts

Samples were analyzed using a Thermo Quest HPLC System (Fremont, CA) equipped with a P2000 binary pump, UV 6000 LP diode array detector, A5 3000 autosampler and Thermo Quest 4.1 chromatography software. Separation was achieved on a C18 column (Alltech Waters Spherisorb ODS-2, 5μm, 250 x 4.6 mm) using a linear gradient system of A: 1% v/v formic acid and B: acetonitrile with 1% formic acid. The operating conditions: injection volume, 20μl; flow rate, 1 ml/min, sample tray at 6°C; column oven at 35°C. The presence of anthocyanins was monitored at 530 nm. An internal standard of cyanindin, 3-5-glucoside was used. Identification of the anthocyanin components was based on retention time. The identity of the anthocyanins was previously determined by LC-MS.

Statistical Analysis

Group differences were evaluated using t-tests with $p<0.05$ considered to be a statistically significant difference. Means were compared with Duncan's multiple range test with $\alpha = 0.05$ using the SAS Statistical Analysis System v8.1 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSIONS

Total Anthocyanin Content

The anthocyanin content of the purple-fleshed sweetpotato clones ranged from 12.3 to 162 mg /100 g fwb for the raw samples. This was greater than the range of 5.3-53.6 mg/100g fwb reported for several purple-fleshed sweetpotatoes in Japan (Furuta et al., 1998). The differences can be attributable to variation in clones, environmental conditions and analytical methods. For cooked samples total anthocyanins ranged from 53 to 281 mg/ 100 g fw. The cooked samples averaged about 1.5 fold higher anthocyanin content than the raw samples. Apparently, the effect of heat treatment on the amount of anthocyanins extracted varied with genotype.

Similar to sweetpotatoes, different varieties of fruits, grains and vegetables also have different levels of anthocyanins content. Abdel-Aal and Hucl (1999) reported that the total anthocyanin in wheat grain varies from 1.04 to 4.58 mg/100g fw. Liu et al., (2002) reported that different varieties of raspberry expressed different levels of anthocyanin content. The reported anthocyanin content of different varieties of raspberries ranged from 0.17 to 57.6 mg anthocyanins/100 fw. Similarly, the anthocyanin content of red potato and red radish also varied within cultivars, ranging from 15-45 mg/100g mL for each commodity (Rodriguez-Soana et al., 1998). These studies also indicated that the expression of pigments and bioactive compounds in plants are due to genetics, maturity and environmental factors (Raffo et al., 2002).

Except for a few samples, most of the total anthocyanin contents for the steamed sweetpotato samples were higher than those of the raw samples (Figure 1). The results are in agreement with previous reports on other fruits and vegetables. Thermal treatment such as UV-light elevated the anthocyanin contents in the apple extracts (Reay and Lancaster, 2001). Heat treatment inactivates enzymes such as phenol oxidase that can catalyze anthocyanin degradation and may also disrupt cell walls which could facilitate release of anthocyanins from cells. Besides that, a phenomenon known as copigmentation of the colored flavylium cation by phenolic substances such as flavonoids or phenolic acids might be possible. Self-association of the flavylium cation, which forms a hydrophobic core surrounded by hydrophilic glycosyl, probably occurs. The phenolic components react with the flavylium cation by using hydroxyl groups. Because of these reactions, the stability of colored flavylium cation is increased, therefore it become more tolerant of thermal processing (Asen et al., 1972). In addition, Inami et al. (1996) reported that isomerization of certain anthocyanins could also increase the stability of the compound. Acylated anthocyanins found in Elderberry (*Sambacus nigra*) could change to a more stable isomers under light irradiation thus increasing the stability of anthocyanins (Inami et al., 1996). Further studies on this area are required for sweetpotato anthocyanins.

Total phenol Content

The total phenolic content ranged from 30.8 to 136.0 mg CAE/100g fwb for the raw samples (Figure 2). After heat treatment, the phenolic content of the

steamed roots of all clones were higher compared to the raw samples (p<0.05). The phenolic content of steamed storage roots ranged from 90.4 to 240.0 mg CAE/100g fwb. There were no significant differences in the dry matter content of the raw and steamed samples (Table 2), but steaming resulted in a 2-3 fold increase in total phenolic contents in the evaluated clones (Figure 2). Cultivar PUR 04-134 and PUR 04-118 had the highest phenolic content among all the raw and steamed samples.

Liu et al. (2002) reported that the phenolic content of raspberry varied among varieties, ranging from 359 to 513 mg gallic acid/100g fw. Gil et al. (2002) reported that different varieties of nectarine, peach, and plum contained different levels of phenolic compound ranging from 138-1403, 172-1202 and 220-3180 mg/kg fwb respectively. These results showed that for any given fruit or vegetable, the phenolic contents can vary significantly among different varieties. This could be due to the genetic make up of different genotypes, which lead to different levels of phenolic expression. On the other hand, the biosynthesis of bioactive compounds could be influenced by environmental factors such as abiotic stress, nutrients, weather, water supply and other growing conditions (Fernando Reyes and Cisneros-Zevallos, 2003).

As with anthocyanins, heat treatment increased the extractable phenolic compounds in the purple-fleshed sweetpotato clone. One possible explanation for the elevation of phenolic content after thermal processing was due to liberation of phenolics from the matrix within the cells in the plant (Gahler et al., 2003). Jeong et al. (2004) also pointed out that phenolic compounds in citrus

peels could be liberated by heat treatment. However, previous studies showed that simple heat treatment could not cleave covalently bound phenolic compounds from rice hulls. However, far-infrared radiation could cause release of covalently bound components. This implied that phenolic compounds of plants could be present in free and bound forms depending on species, and that effective processing steps forms for liberating phenolic compounds may vary among plant materials (Jeong et al., 2004).

Oxygen Radical Absorbance Capacity (ORAC)

The result of the ORAC analysis of the antioxidant activities of hydrophilic extracts of the purple-fleshed sweetpotato indicated that the antioxidant activity also varied significantly ($p<0.05$) within the purple-fleshed sweetpotato materials (Figure 3). The antioxidant activity of most samples was centered between 10-25 μM TE/ g fwb for both raw and steamed samples. The antioxidant activity of the raw samples ranged from 3.10 to 31.1 μM TE/ g fwb, whereas the antioxidant activity of the steamed sample ranged from 7.00 to 40.5 μM TE/ g fwb.

A wide range of antioxidant activity in different varieties of other fruits and vegetables has been reported. The ORAC antioxidant capacity that were reported for cranberry, lingonberry, blueberry and chokeberry were 9.2, 12.8, 22.8 and 95.1 μM TE /g fwb respectively (Zheng et al., 2003). For broccoli, the ORAC values of the hydrophilic fractions varied from 38.1 to 121 μM TE/g dw (Kurilich et al., 2002). The antioxidant activity for brown sorghum and black sorghum obtained from ORAC procedure were 878 and 271 μM TE/g dw, respectively (Akiwa et al., 2005).

Thermal processing elevated the antioxidant activities of most sweetpotato cultivars, but the increase was not as large as was observed for anthocyanin and phenolic compounds. Other types of fruits and vegetables also exhibited increases in antioxidant activities as affected by thermal processing. The antioxidant activity of citrus peels increased as the heating temperature increased. This could be explained by newly formed low molecular weight phenolic compounds in the citrus peel extract when it was heated at high temperature, approximately 150 ^{0}C (Jeong, 2004). However, Philpott et al. (2003) reported that the antioxidant activity and anthocyanins content of purple-fleshed sweetpotatoes were not affected by common cooking methods.

There were high correlations between the ORAC values and total phenol contents for both the raw (R^2=0.764) and steamed (R^2=0.661) samples [Fig 4(a) & 4(b)]. However, the correlation between ORAC values and total anthocyanins for both raw (R^2=0.380) and steamed (R^2=0.233) samples was poor [Figure 5(a) & 5(b)]. Previous results (Chapter 2) showed a better correlation coefficient (R^2=0.500) between ORAC values and a wide range of anthocyanin concentrations in raw samples of seven sweetpotato clones. It can be speculated that, aside from the quantity, the composition of the extracted anthocyanins may have different effects on antioxidant activities. In a study on radical scavenging activity of several purple-fleshed sweetpotato cultivars, Oki et al. (2002) found that anthocyanins and phenolic compounds have different contributions to the antioxidant activity irrespective of the purple shade of the cultivars evaluated. Philpott et al. (2003) indicated that in addition to anthocyanins, hydroxycinnamic

acid responsible for some of the antioxidant activity in purple-fleshed sweetpotatoes.

There was a correlation between phenolic compounds and the anthocyanins concentration in the raw samples of 0.741 (Figure 6 (a)). This indicated that the phenolic content and anthocyanin content were parallel to each other in the raw samples. However, after thermal treatment, there was a poor correlation (R^2=0.191) between phenolic compounds and anthocyanins content (Figure 6(b)). Further studies are required to better understand the effects of heat treatment on the antioxidant activities and changes in phenolic components and anthocyanin compounds in purple-fleshed sweetpotato.

HPLC Analysis of Sweetpotato Anthocyanins

A typical HPLC profile of anthocyanins is shown in Figure 7. The major peaks were separated, and the identities of eight major peaks were identified by LC-MS in our previous work. Peaks labeled as A, B, C and E cyanidin anthocyanins, whereas peaks labeled D, F, G and H are peonidin anthocyanins. Even though all the samples had purple flesh color, the ratio of peonidin to cyanidin varied among the cultivars, ranging from 0 to 53.2 and 0.05 to 14.8 for raw and steamed root respectively (Table 1). Based on this ratio, the anthocyanins in those cultivars could be categorized as either cyanidin or peonidin-rich type. Variation in the anthocyanin compositions among different cultivars of purple-fleshed sweetpotato has been reported. Yoshinaga and coworkers (1999) showed that ten of the nineteen cultivars analyzed had peonidin as the major anthocyanins in the flesh of the root (peonidin/cyanidin

ratio of 0.02 to 24.62). At the same time, Oki et al. (2002) did a HPLC analysis of anthocyanins and the results showed that two of the five samples were rich in peonidin aglycon and the other three cultivars were rich in cyanidin aglycon.

Color Analysis of Paste and Powder

The results of color analysis indicated that the color of the samples ranged from dark purple to bluish purple, and lightness of purple color also varied among the cultivars. The L*, a* and b* values for both raw and cooked samples varied among varieties (Table 2). There was a range of b* value for both raw and cooked samples, but they were all negative values indicating that the samples were purple/bluish color. The a* value for all samples were positive values indicating that the samples were reddish in color.

In this study, the pH was relatively constant and close to pH 7 for all the paste samples, so pH would not substantially affect paste color. Based on plot of L* versus b*/a* samples in the powdered form had a higher value of L* compared to paste. This indicated that the color of powder was brighter relative to the paste. The L* values of the powders were in the range of 20-60, whereas the L* values of the pastes were in the range of 10-30 (Table 2). Yoshinaga et al., (1999) and Oki et al. (2002) reported similar results for the purple-fleshed sweetpotatoes. Visually, the samples were noticeably darker, lower L* value, after heat treatment (Figure 8). The paste had a wider range of b*/a* value as compared to the powdered samples. The range of b*/a* values was -0.0296 to -0.788, and -0.032 to -0.473 for the paste and powder, respectively. According to Yoshinaga et al. (1999), the combination of HPLC and color analysis results

revealed that samples with a b*/a* value greater than -1.1 had peonidin as the major anthocyanin. While, samples with a b*/a* value or less than -1.4 had primarily cyanidin anthocyanins. All purple-fleshed sweetpotatoes were peonidin-rich based upon a b*/a* ratio above -1.0 (Figure 9). However, HPLC analysis showed that anlysis on the basis of the b*/a* ratio was often incorrect in the prediction of the major anthocyanin (Table 1).

Conclusions

The results show that the anthocyanin content, phenolic content and antioxidant activity varied over a wide range in this collection of purple-fleshed sweetpotatoes. Heat treatment of the sweetpotatoes increased the amount of anthocyanins, phenolic compounds, and antioxidant activity extracted. HPLC analysis of the anthocyanins showed that the peonidin/ cyanidin ratio did not accurately predict the major anthocyanin in the sweetpotatoes.

Future Studies

Over all the results indicated that heat treatment increased the bioactive compounds as well as antioxidant activity of purple-fleshed sweetpotatoes. However, thermal processing of this study was limited to steaming. Future study should include various heat treatment techniques to determine if the effect would be the same as steaming.

HPLC analysis was a more precise technique to quantify the anthocyanin compositions of sweetpotatoes compared to qualitative color analysis procedure. Future studies on the anthocyanin compositions of the storage roots should be based on HPLC analysis.

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Table 1: Anthocyanins compositions in raw and steamed sweetpotato clones

Samples	no.*	Percent of Pigments (Raw)		Peo: Cya ratio (Raw)	Percent of Pigments (Steamed)		Peo:Cya ratio (Steamed)
		Peo (Red)	Cya (Blue)		Peo (Red)	Cya (Blue)	
PUR 04-118	1	89.4	3.1	29.22	34.3	23.7	1.45
PUR 04-113	2	22.2	61.8	0.36	22.4	63.2	0.35
PUR 04-073	3	84.7	1.6	53.21	4.9	93.4	0.05
PUR 04-076	4	82.9	12.7	6.50	56.3	35.4	1.59
PUR 04-062	5	55.9	19.2	2.91	45.5	33.9	1.34
PUR 04-078	6	29.3	54.2	0.54	24.6	60.1	0.41
PUR 04-106	7	78.6	7.2	10.97	56.0	23.9	2.34
PUR 04-127	8	57.9	34.8	1.66	85.0	5.8	14.79
PUR 04-128	9	26.5	50.0	0.53	19.7	35.6	0.55
PUR 04-136	10	34.8	57.6	0.60	36.6	51.2	0.71
PUR 04-087	11	83.4	16.6	5.02	63.0	27.6	2.28
PUR 04-134	12	64.8	17.9	3.62	27.6	55.0	0.50
PUR 04-120	13	79.6	20.4	3.90	40.1	42.2	0.95
PUR 04-122	14	100.0	0.0	0.00	18.3	57.9	0.32
PUR 04-130	15	0.0	50.9	0.00	46.3	45.4	1.02
PUR 04-126	16	0.0	81.0	0.00	25.1	49.9	0.50
PUR 04-135	17	0.0	92.4	0.00	56.0	35.0	1.60
PUR 04-146	18	45.5	33.2	1.37	37.5	53.7	0.70
PUR 04-147	19	0.0	37.0	0.00	30.2	49.5	0.61
PUR 04-139	20	0.0	86.4	0.00	14.9	69.0	0.22
PUR 04-144	21	2.4	94.6	0.03	35.6	56.5	0.63

***Sample number, Peo = peonidin, Cya=cyanidin**

Table 2: Dry matter content, L*, a* and b* values of raw and steamed sweetpotatoes.

Sample Name	No.*	pH	Raw Samples				Steamed Samples			
			Dry matter, %	L*	a*	b*	Dry matter, %	L*	a*	b*
PUR 04-118	1	6.26	30.0	51.5	51.5	-4.39	31.5	25.2	25.2	-8.12
PUR 04-113	2	645	25.9	34.7	34.7	-0.63	26.2	17.4	17.4	-2.73
PUR 04-073	3	6.86	25.9	22.4	22.4	-2.65	26.4	18.9	18.9	-12.25
PUR 04-076	4	6.24	31.3	50.4	50.4	-4.55	31.1	12.2	12.2	-7.91
PUR 04-062	5	6.21	29.7	37.8	37.8	-3.50	30.2	18.6	18.6	-8.88
PUR 04-078	6	6.70	25.0	38.4	38.4	-6.47	25.8	16.0	16.0	-2.78
PUR 04-106	7	6.3	26.5	50.5	50.5	-3.43	27.8	20.9	20.9	-9.90
PUR 04-127	8	6.37	33.2	52.6	52.6	-1.91	37.0	18.8	18.8	-0.74
PUR 04-128	9	6.58	30.7	50.6	50.6	-4.68	29.2	21.2	21.2	-9.97
PUR 04-136	10	6.87	29.0	51.8	51.8	-4.85	30.6	16.6	16.6	-8.01
PUR 04-087	11	6.45	28.0	51.5	51.5	-1.70	27.3	16.0	16.0	-12.16
PUR 04-134	12	6.35	32.4	51.6	51.6	-2.26	32.4	19.1	19.1	-0.29
PUR 04-120	13	6.10	22.4	51.5	51.5	-4.70	25.1	15.2	15.2	-9.04
PUR 04-122	14	6.54	28.8	51.6	51.6	-6.46	30.6	20.8	20.8	-3.21
PUR 04-130	15	6.89	28.6	50.6	50.6	-5.53	28.5	18.5	18.5	-8.33
PUR 04-126	16	6.31	36.4	51.4	51.4	-4.45	35.3	27.8	27.8	-14.63
PUR 04-135	17	6.35	41.1	45.6	45.6	-8.65	33.8	20.5	20.5	-9.01
PUR 04-146	18	6.48	37.0	44.5	44.5	-9.75	35.6	18.8	18.8	-10.03
PUR 04-147	19	6.89	28.3	40.6	40.6	-4.43	32.7	18.2	18.2	-13.25
PUR 04-139	20	6.46	33.3	45.5	45.5	-4.59	30.9	13.6	13.6	-7.36
PUR 04-144	21	6.36	40.5	45.5	45.5	-5.72	38.5	25.5	25.5	-9.13

*** Sample number.**

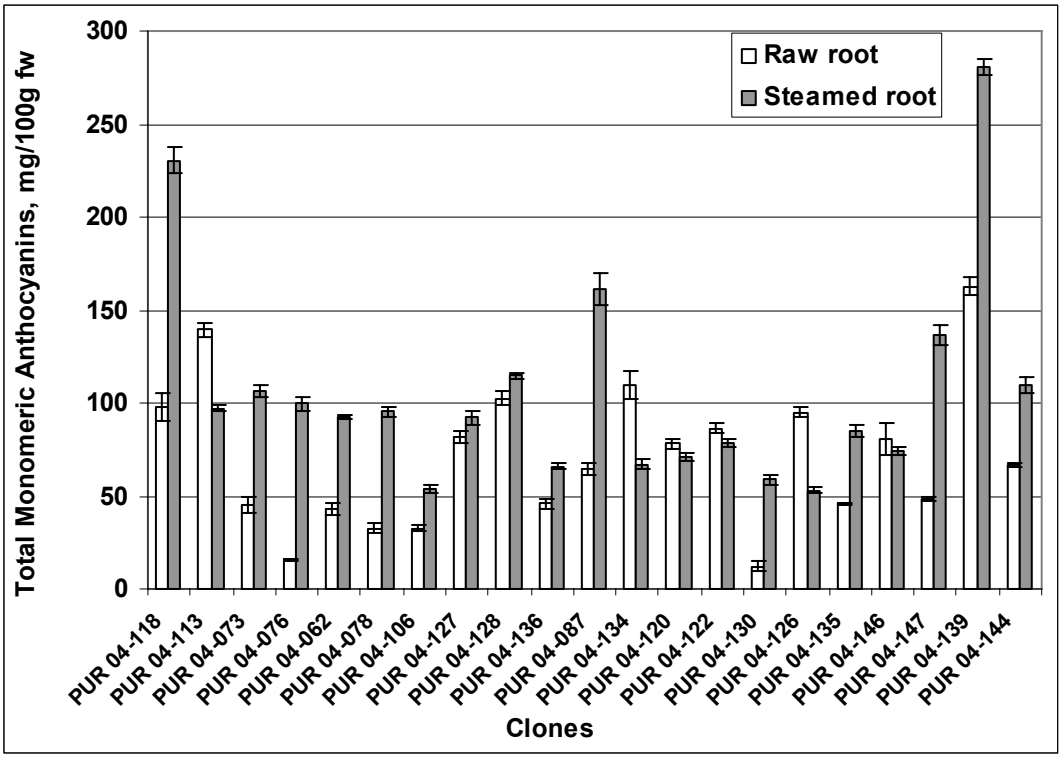


Figure 1: Total anthocyanins of raw and steamed purple-fleshed sweetpotato clones.

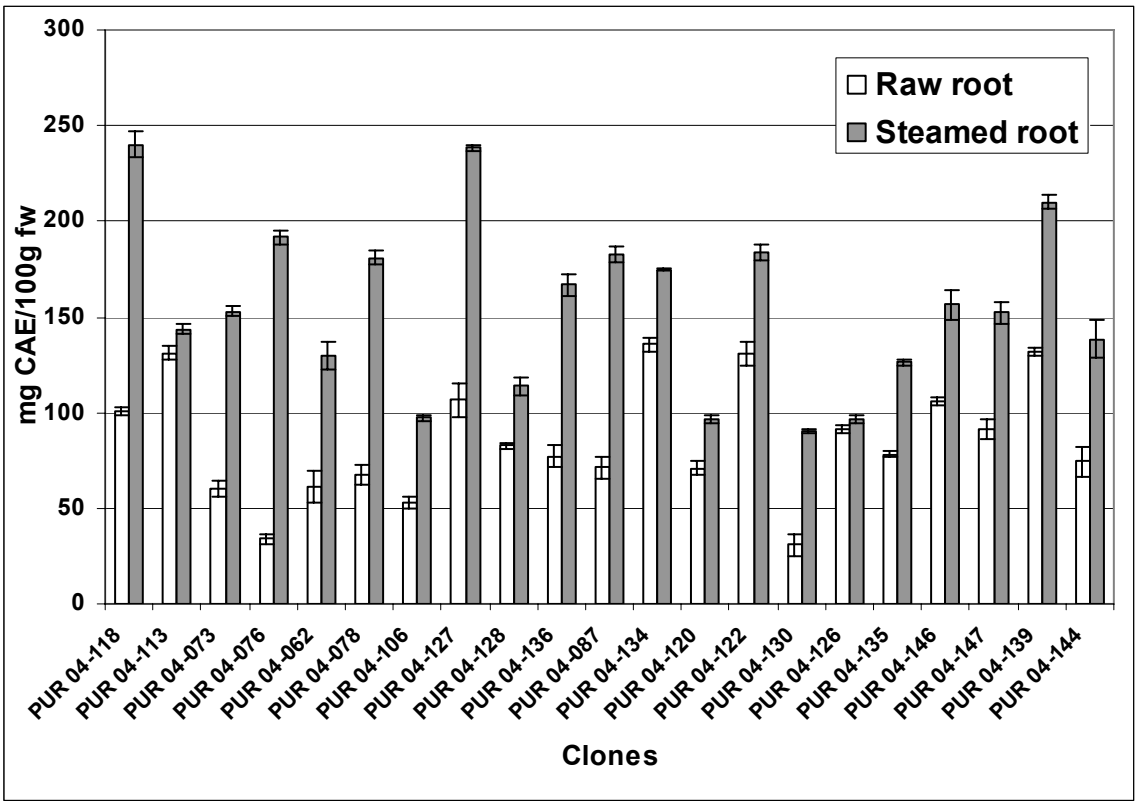


Figure 2: Total phenolic contents of raw and steamed purple-fleshed sweetpotato clones.

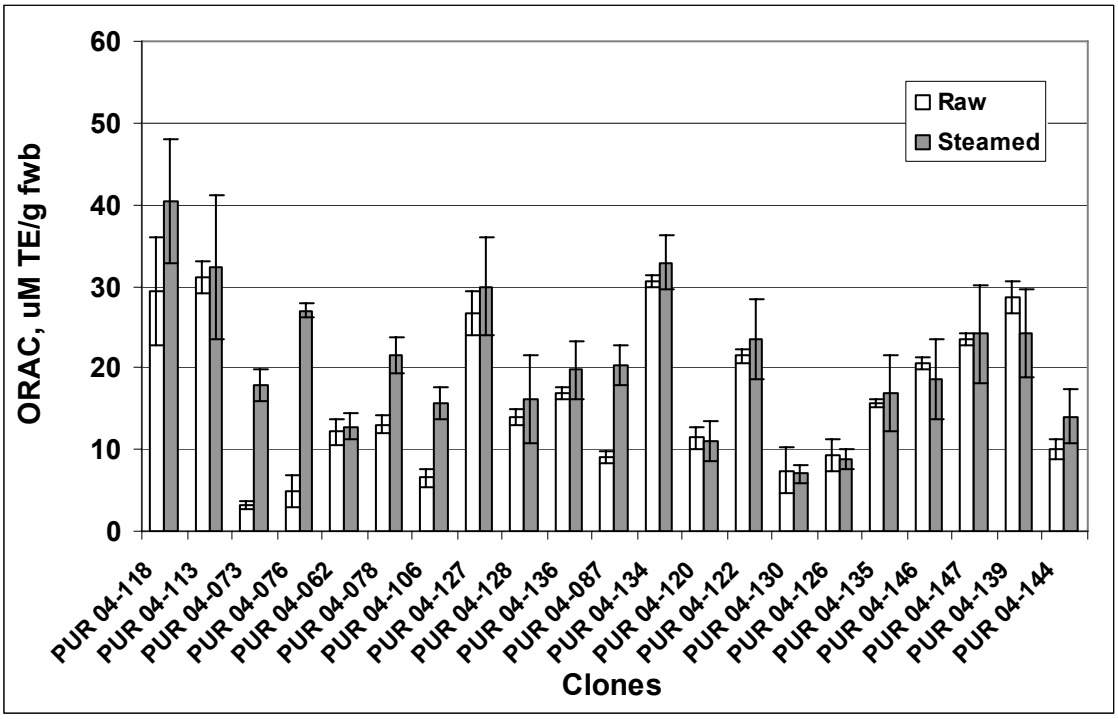


Figure 3: ORAC-Total antioxidant activity of raw and steamed purple-fleshed sweetpotato clones

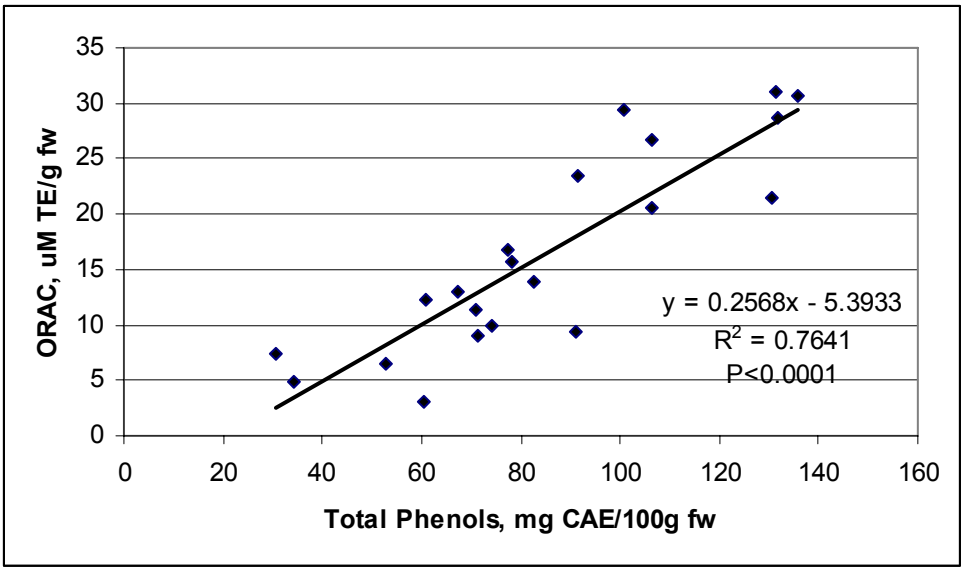


Figure 4(a): Correlation between phenolic compounds and ORAC in raw purple-fleshed sweetpotato clones

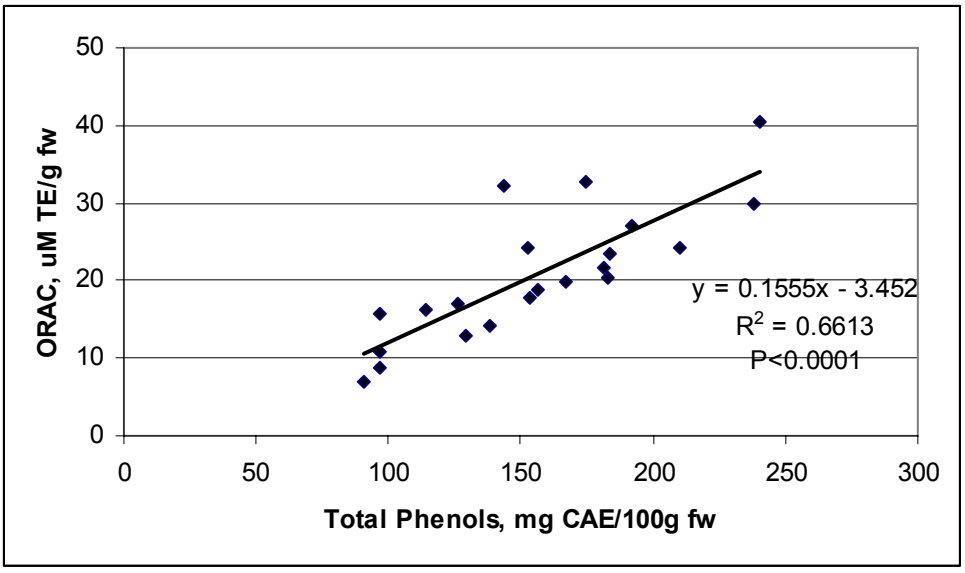


Figure 4(b): Correlation between phenolic compounds and ORAC in steamed purple-fleshed sweetpotato clones.

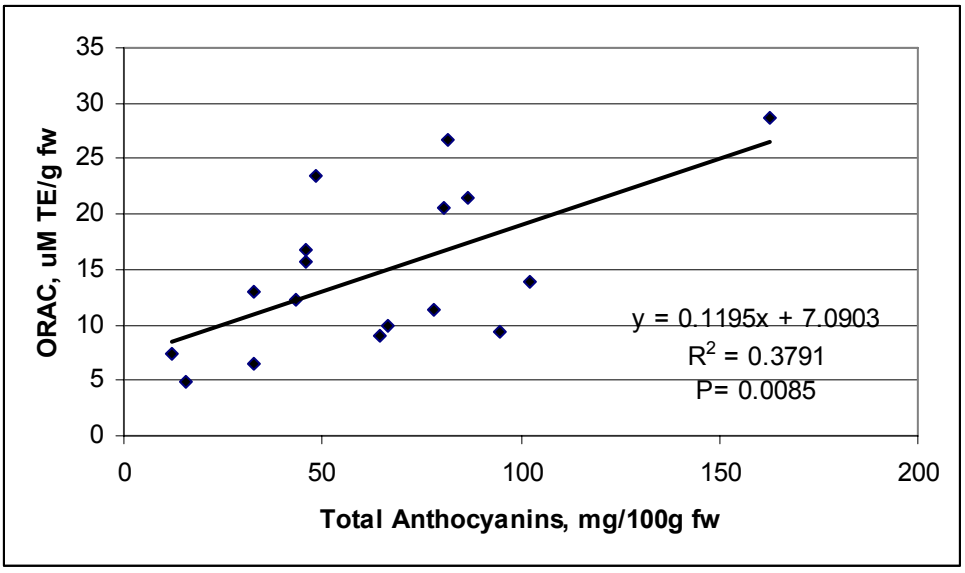


Figure 5(a): Correlation between total anthocyanins and ORAC in raw purple-fleshed sweetpotato clones.

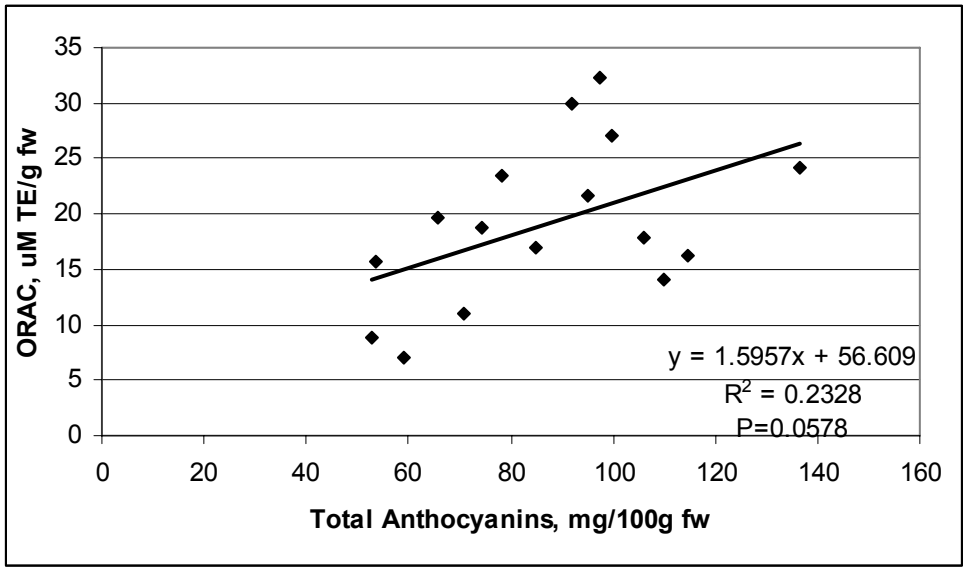


Figure 5(b): Correlation between ORAC and total anthocyanins in steamed purple-fleshed sweetpotato clones.

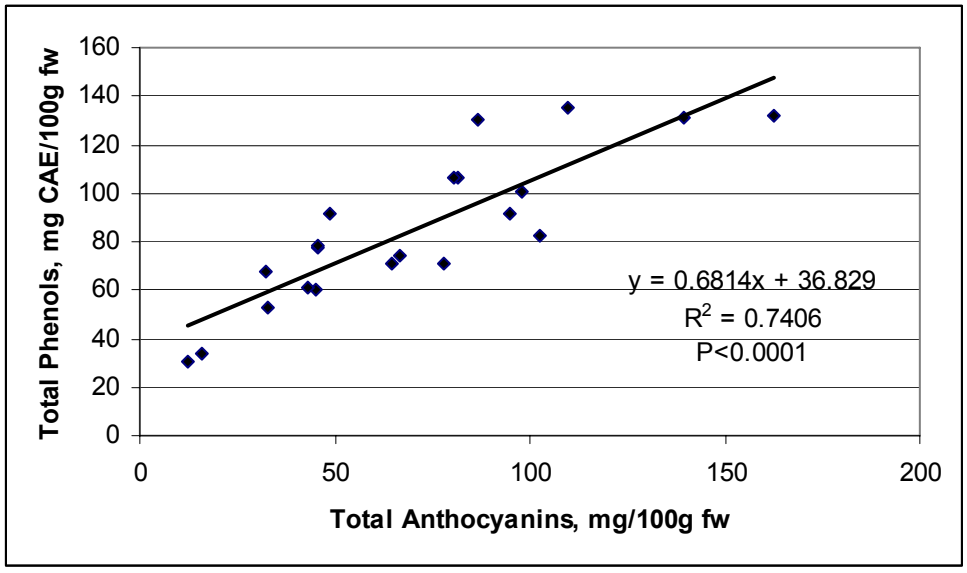


Figure 6(a): Correlation between total anthocyanins and phenolic compounds in raw purple-fleshed sweetpotato clones.

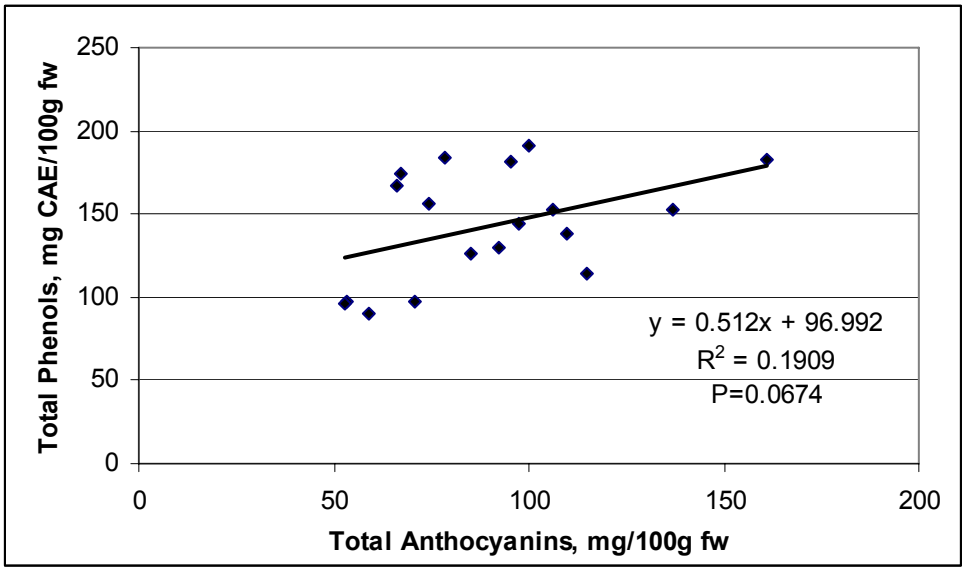


Figure 6(b) Correlation between total anthocyanins and phenolic compounds in steamed purple-fleshed sweetpotatoes.

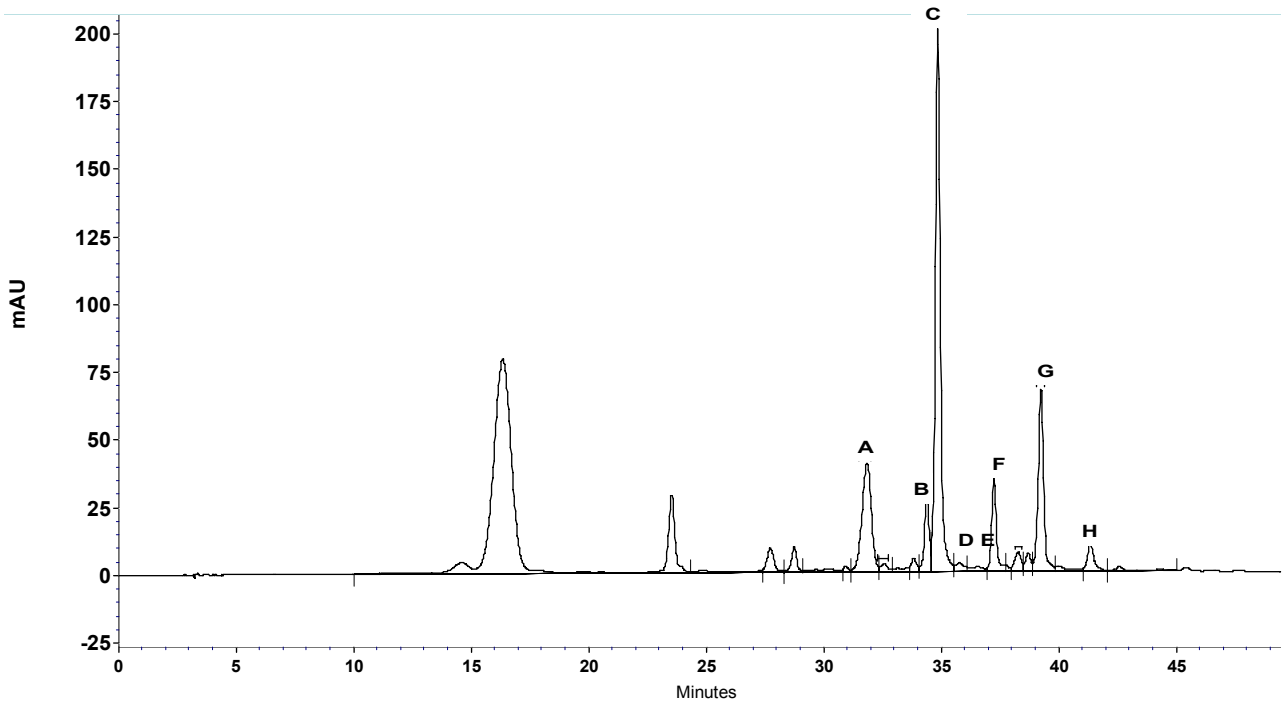
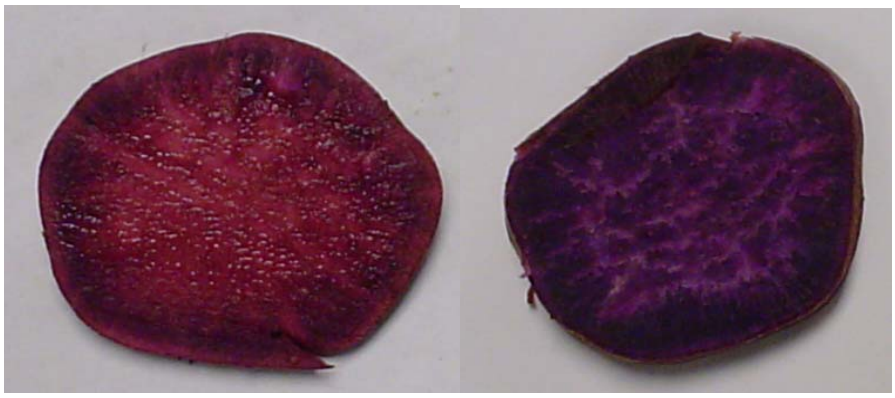


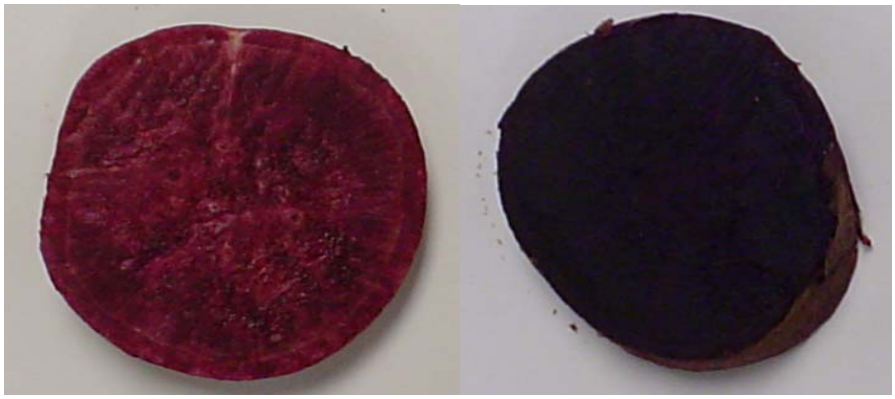
Figure 7: HPLC chromatogram of anthocyanins for clone PUR 04-113
Peaks A, B, C and E are cyanidin, peaks D, F, G and H are peonidin.

PUR 04-118



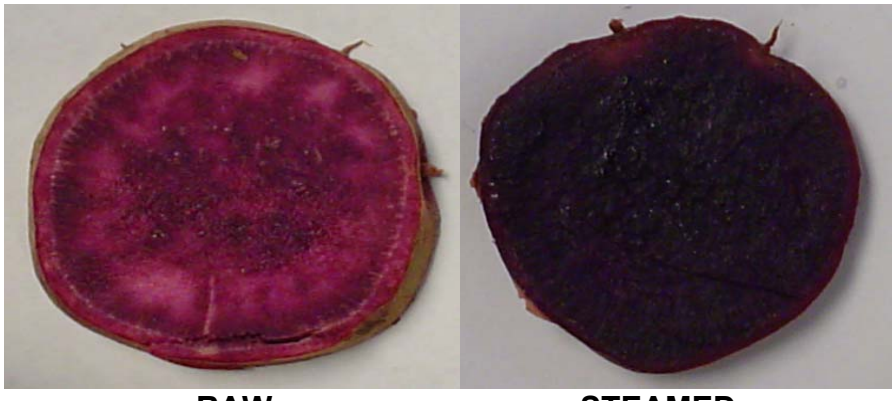
RAW **STEAMED**

PUR 04-076



RAW **STEAMED**

PUR 04-106



RAW **STEAMED**

Figure 8.0: Comparison of color differences between raw and steamed sweetpotatoes

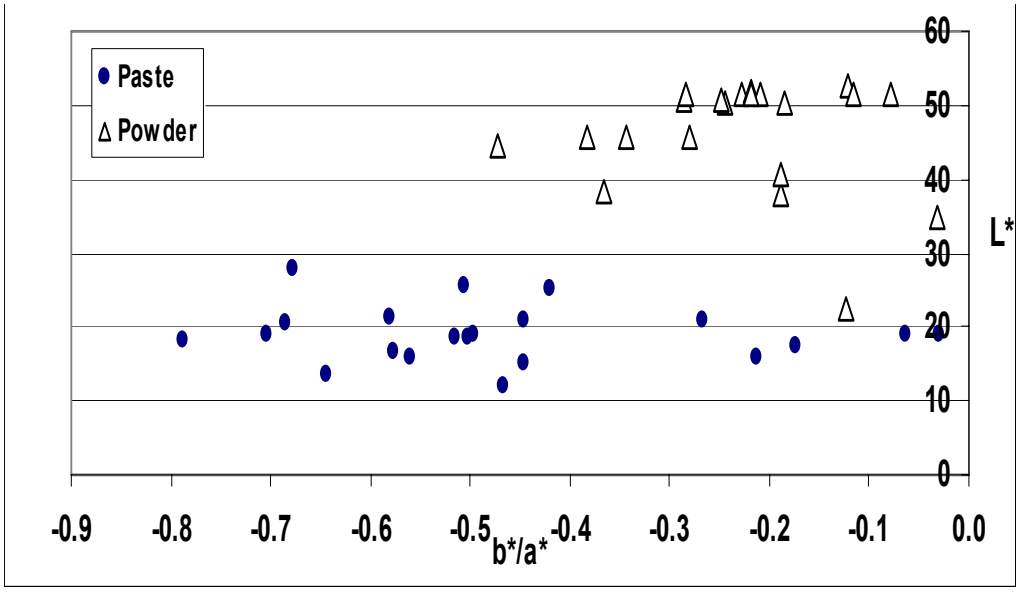


Figure 9: Reflectance value of raw and steamed purple-fleshed sweetpotato clones.