Identification and Quantification of Anthocyanins in Sorghum and Sweetpotato Leaves

by

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B.S., Kansas State University, 2012
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AN ABSTRACT OF A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Food Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2018

Abstract

Anthocyanins are the largest group of water-soluble pigments within fruits and vegetables. The potential health benefits of plant-derived anthocyanin-rich foods have attracted much attention. This dissertation focused on identification and quantification of anthocyanins in sorghum and sweetpotato leaves.

Study 1: Sorghum is a rich source of various phytochemicals including anthocyanins. This study was to identify and quantify the profiles of anthocyanins by HPLC-DAD in the selected 25 sorghum accessions with various phenotypic pericarp pigments. The predominant anthocyanins found in sorghums were 3-deoxyanthocyanidins including the unique leuteolinidin and apigeninidin analogs. The high levels of total anthocyanins were found in the red pericarp PI297139 and the brown pericarp PI221723, followed by the brown pericarp PI35038 and the yellow pericarp PI229838. There were moderate to low levels of anthocyanins observed in all the other accessions except for the white pericarp that generally contained least to undetectable amount. Although anthocyanins appeared to be associated with the pericarp color in the sorghum accessions with the highest contents in each pericarp pigment category, a distinguishable diversity of anthocyanin contents was presented among and between the phenotypic pericarp colors, suggesting other colorful phytochemicals such as carotenoids might be contributed. Establishing a database of anthocyanin profile and diversity in sorghum accessions with various pericarp pigments may lead to the development of novel functional sorghum products with active anthocyanin-enriched health benefits.

Study 2: As phytochemical-enriched edible greens, sweetpotato (*Ipomoea batatas L.*) leaves have become popular. However, the profile and content of phytochemicals in sweetpotato leaves are mostly unknown. We previously bred a purple-fleshed sweetpotato P40 that

demonstrated cancer prevention due to high levels of anthocyanins in the tuberous roots. The objectives of this study were to identify and quantify anthocyanins in P40 leaves when compared with the white-fleshed Bonita and orange-fleshed Beauregard. The mature leaves of P40 at 6-week vine stage were collected and extracted for anthocyanin analysis by HPLC-MS/MS. Fourteen anthocyanins, including a novel anthocyanin (peonidin 3-caffeoyl-p-coumaryl sophoroside-5-glucoside), were identified and quantitated. The contents of anthocyanins in P40 leaves (38 ± 2.9 mg/kg DW) were much lower than that in tubers (13,100 ± 70 mg/kg DW). Furthermore, anthocyanin contents in P40 leaves were even lesser than those of the white-fleshed Bonita (448 ± 50.4 mg/kg DW) and orange-fleshed Beauregard (240 ± 60.9 mg/kg DW). Total phenolic contents as measured by Folin-Ciocalteu were 36.8 ± 4.8 mg GAE/g DW in the leaves of P40, but 46.7 ± 2.1 mg GAE/g DW in Bonita and 41.2 ± 5.0 mg GAE/g DW in Beauregard. No anthocyanin was detectable in the stems of these three sweetpotato varieties. Taken together, this study reports for the first time the profile and content of anthocyanins in the leaves of three sweetpotato varieties with a new anthocyanin identified. The unexpected lower levels of anthocyanins in the purple-fleshed sweetpotato leaves when compared with the tuberous roots advanced our existing database and also validated a diverse genotype of anthocyanin biosynthesis between sweetpotato leaves and tubers.

Study 3: As phytochemical-enriched edible greens, sweetpotato (Ipomoea batatas L.) leaves have potential health benefits. However, how anthocyanin content in sweetpotato leaves responds to harvest stages and growth conditions remains mostly unknown. In this study, we investigated the effect of harvest timing on the accumulation of anthocyanin in the leaves of several sweetpotato varieties: white-skinned and white-fleshed Bonita, red-skinned and orange-fleshed Beauregard, red-skinned and white-fleshed Murasaki, and purple-skinned and purple-

fleshed P40. Anthocyanin content increased continuously in Bonita from 1st slip stage to vine stage, but P40 did not have the same response. Beauregard had most anthocyanin (592.5 ± 86.4 mg /kg DW) and total phenolic content (52.2 ± 3 mg GAE/g DW) of mature leaves at vine stage. The P40 variety had low anthocyanin and total phenolic content in the leaves although P40 tubers have the highest among these varieties. In the high tunnel studies, no significant differences in anthocyanin content were found in Beauregard leaves grown in the high tunnels versus the open field. Our study showed for the first time that anthocyanin levels were significantly affected by the growth stages. Our overall results indicate that growth stage and/or environmental factors among sweetpotato varieties affected anthocyanin content, which is highly variable and genotype-dependent.

In conclusion, the three studies conducted in this dissertation provided a fundamental understanding of anthocyanin profiles and contents in various sorghum accessions with various phenotypic pericarp pigments and sweetpotato leaves in various growth stages and conditions. These results can be useful not only for the breeders but also consumers in the selection of sorghum accessions and sweetpotato varieties for anthocyanin-contained health benefits.

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Approved by:

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Abstract

Anthocyanins are the most abundant water-soluble flavonoid pigments that are biosynthesized via the phenylpropanoid pathway in plants. Consumption of anthocyanin-rich vegetables and fruits has been linked with multiple health benefits in chronic disease prevention. This dissertation consisted of three studies as follows focused on the profiles and contents of anthocyanins in various sorghum accessions and sweetpotato leaves.

Study 1: Sorghum is a rich source of various phytochemicals, but the contents of anthocyanins in various sorghum accessions are not clear. This study was to identify and quantify the anthocyanins by HPLC-DAD in selected 25 sorghum accessions with various phenotypic pericarp pigments. The predominant anthocyanins found in sorghums were 3-deoxyanthocyanidins including the unique leuteolinidin and apigeninidin analogs. The high levels of total anthocyanins were found in the red pericarp PI297139 and the brown pericarp PI221723, followed by the brown pericarp PI35038 and the yellow pericarp PI229838. There were moderate to low levels of anthocyanins observed in all the other accessions except for the white pericarp that generally contained least to undetectable amount. Although anthocyanins appeared to be associated with the pericarp color in the sorghum accessions with the highest contents in each pericarp pigment category, a distinguishable diversity of anthocyanin contents was presented among and between the phenotypic pericarp colors, suggesting other colorful phytochemicals such as carotenoids might be contributed. Establishing a database of anthocyanin profile and diversity in sorghum accessions with various pericarp pigments may lead to the development of novel functional sorghum products with active anthocyanin-enriched health benefits.

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In conclusion, the three studies conducted in this dissertation provided a fundamental understanding of anthocyanin profiles and contents in various sorghum accessions with various phenotypic pericarp pigments and sweetpotato leaves in various growth stages and conditions. These results can be useful not only for the breeders but also consumers in the selection of sorghum accessions and sweetpotato varieties for anthocyanin-contained health benefits.

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Acknowledgements

First and foremost, this research project would not have been possible without the support of many people. I would like to express my gratitude to all of those who gave me the possibility to complete this dissertation. I wish to express my sincere gratitude to my major Professor, Dr. Weiqun Wang, who inspired me in my dissertation project. I would also like to thank my supervisory committee including Dr. Weiqun Wang, Dr. Cen Wu, Dr. Sunghun Park, Dr. Fadi Aramouni, and Dr. Sajid Alavi for their time and valuable advice. I am especially thankful to Dr. Jason Griffin at K-State Horticultural who bred the sweetpotatoes for my dissertation project.

My completion of this project also could not have been accomplished without the support of my classmates, Mrs. Miao Yu, Dr. Xi Chen, Mrs. Yanting Shen, and Dr. Jingwen Xu.

I would like to give my special thanks and express my love and gratitude to my wife and daughter for their understanding and endless love, through the duration of my dissertation studies.

Dedication

Dedicated to my parents, Yongjia Su and Chunmei Ding, for your endless love, encouragement, and support.

Preface

Chapter 2 was formatted for publication according to the required standards of the journal: Journal of Nutrition & Food Sciences. Chapter 3 was formatted for publication according to the required standards of the journal: Food Chemistry. Chapter 4 was formatted for publication according to the required standards of the journal: Journal of Agricultural and Food Chemistry.

Chapter 1 - Literature review

Summary

Antioxidant phytochemicals like phenolics and flavonoids are secondary plant metabolites constructed with one or more hydroxylated aromatic or phenolic rings. These chemical compounds are synthesized along the shikimate pathway through L-phenlylalanine.[1] A variety of phytochemicals are created along this synthesis pathway that has deamination, enzymatic conversion, and enzymatic hydroxylation reactions.[2] Depending on the number of phenolic rings in the structure, phytochemicals can be sorted into a single phenolic ring category (e.g., phenolic acid) or two or more phenolic rings (e.g., polyphenolics and tannins). Polyphenolics are a class of flavonoids classified into isoflavones, flavones, flavonols, flavones, flavanones, and anthocyanins. Isoflavones are found in beans, while flavonol is common in apples. Tea and coffee contain flavanol and catechin. Tannins, as polymerized polyphenols, are found in sorghum, fruit juice, and red wine.[3, 4] Anthocyanins are common in vegetables and fruits, and phenolic backbones can also have varying side chains like sugar, long carbon chains, and phytosterols that lead to different phenolic derivatives.[5] Many phenolic derivative phytochemicals are important in helping plants resist oxidative and environmental stresses like UV radiation, microbes, pathogens, and parasites.[6, 7]

Anthocyanins (derived from Greek *Anthos* (flower) and *kyanos* (dark blue)) are an important water-soluble plant pigment. The significant property of anthocyanin, studied in depth recently, is its role in preventing cardiovascular diseases, cancer, and diabetes.[8-12] Therefore, increasing interest and research on anthocyanin focus on its potential health benefits, developing purification and extraction methods, discovering its biosynthesis pathway, and identifying and quantitatively analyzing it using HPLC-MS.[13-16] As one of the most important water-soluble

plant pigments, anthocyanins contribute to blue, purple, or red color of many plants.[14] They are synthesized along the flavonoid branch of the phenylpropanoid pathway through secondary metabolism in higher plants. Among the more than 600 types of anthocyanins, most anthocyanin aglycones found in nature consist of six anthocyanidins: cyaniding (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), pelargonidin (Pg), and malvidin (Mv).[15] These six share a 2-phenylbenzopyrilium (flavyl-ium) skeleton hydroxylated in 3, 5, and 7 positions, with different substitutions at R_1 and R_2 (see Figure 1-1).

Glucose, galactose, arabinose, rhamnose and xylose are the most common sugars that decorated to anthocyanidins into mono-, di-, or tri- saccharide formats. A series of aromatic and aliphatic acid are normally applied to acylate sugar moieties, such as cinnamic acid[16, 17] (Figure 1-2). In solution, the anthocyanin transmits in equilibrium with essentially four forms: the flavylium cation, the quinoidal base, the hemiacetal base, and chalcone.[18] The proportion of these four forms can vary due to pH and structure of anthocyanins.[19-21] In comparison with other flavonoids, anthocyanins presents primarily as the stable flavylium cation when the pH is less than 2 in terms of a positive charge on its C-ring, which leads to different colors in response to various pH.[22]

This review falls into two parts: 1) the first part reviews the background information of the characteristics of anthocyanins presented in sorghum, associated with gene expression mechanisms and analytical methods; 2) the second part introduces anthocyanin profiles and contents in sweetpotato leaves through different analytical methods.

Part 1 Anthocyanins in sorghum, their gene expression and analytical methods

Background

Sorghum [*Sorghum bicolor (L.) Moench*] ranks fifth among staple crops in the world for grain production; it is a good source of protein, calories, and minerals for millions of people in the warmer climates and tropical regions like Asia and Africa.[23-25] The United States is the largest producer and exporter of sorghum, with 20% of total world production and approximately 80% of world sorghum exports in 2001-2003.[26-27] In many developing countries, sorghum is a food item.[28]

Anthocyanins in sorghum

Among cereals, sorghum contains the most total phenolic content, up to 6% (w/w) in some cultivars.[24] The sorghum kernel comprises four main parts: pericarp (outer covering), testa (layer between the pericarp and endosperm), the endosperm (storage tissue) and the germ or embryo[29, 30] (see Figure 1-3). Phenolic compounds (phenolic acids, flavonoids, and tannins) are most widely distributed in the pericarp and testa of the sorghum grain.[29-31] Of the more than 600 types of anthocyanins, most anthocyanin aglycone found in nature consists of six anthocyanidins: cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin.[15] Sorghum grain is the only known dietary source of 3-deoxyanthocyanidins, which otherwise have only been found in the flowers of sinningia (*Sinningia cardinalis*), the silk tissues of maize (*Zea mays*), and the stalks of sugarcane (*Saccharum sp*.).[32-34] 3-Deoxyanthocyanidins are yellow anthocyanidins with a backbone lacking a hydroxyl group on the carbon 3 position; it is a more

stable anthocyanidin in acidic solutions than other anthocyanidins found in most natural food.[31-34]

Figure 1-4 is a schematic representation of the 3-deoxyanthocyanidin biosynthetic pathway and the potential sites of the expression of the biosynthetic enzymes in sorghum. In sorghum, the biosynthetic pathway for 3-deoxyanthocyanidins deviates from the ordinary anthocyanin pathway by reducing flavanones via a *DFR* enzyme (*dihydroflavonol 4-reductase*), followed by an *ANS* (*anthocyanidin synthase*) reaction step.[35, 36]

Anthocyanidin content

Dietary sorghum with 3-deoxyanthocyanidins are associated with human health benefits like preventing obesity and diabetes through antioxidant and anti-inflammatory mechanisms.[26, 27] Therefore, increasing research interest has developed in 3-deoxyanthocyanidin levels in various sorghum accessions, along with development of purification and extraction methods. In previous studies, 3-deoxyanthocyanidins from black, brown, and red sorghums have been characterized using a variety of analytical methods.[37-40] Among them, the black sorghum brans were a better source of anthocyanins (4.0–9.8 mg/g) than other commercial vegetables and fruits (0.2–10 mg/g).[15] Black sorghum anthocyanins are mainly composed of the 3-deoxyanthocyanidins, which are more stable than the anthocyanins found in fruits.[37-39] In comparison, the brown and red sorghum brans have less 3-deoxyanthocyanin content, ranging from 2.8 to 4.3 mg/g.[38-40] Sorghums with red pericarps have a high proportion of apigeninidin while a purple pericarp indicates a high proportion of luteolinidin, which suggests that secondary plant color may affect 3-deoxyanthocyanin composition.[38]

Gene expression

Of the approximately 45,000 sorghum accessions now in crop gene banks, researchers have become increasingly interested in black and brown sorghum for the 3-deoxyanthocyanidin, which has health benefits. In sorghum, 3-deoxyanthocyanidin is found in the pericarp of mature seed and is associated with the functional yellow seed1 (*recessive allele-y1*) gene.[41, 42] Molecular and genetic analysis indicated a null *y1* allele designated as *y1-ww* (white pericarp, white glume) is not transcribed, which results in a loss of *Y1*-regulated expression of structural genes needed for the biosynthesis of 3-deoxyanthocyanidin. Compared with white sorghum, 3-deoxyanthocyanidins in sorghum dominant allele *Y1-rr* (red pericarp, red glume) were significantly higher in other accessions, while the *y1-ww* extracts not showing the presence of 3-deoxyanthocyanidin indicate that a functional *y1* gene considered as a transcription of *DFR* in tissues can cause accumulation of 3-deoxyanthocyanidin pigment in pericarps.[42] Moreover, previous studies have directly demonstrated that, unlike a functional *Y1-rr* allele, an *y1-ww* deletion line does not lead to accumulation of 3-deoxyanthocyanidin.[41,42]

Analytical methods

Colorimeter

Sorghum samples were visually phenotyped and quantitatively measured for color via colorimeter to estimate grain composition values.[43, 44] Several methods, including colorimetric methods, have been used to determine sorghum 3-deoxyanthocyanidin levels. The colorimeter identifies color using three attributes: L* (lightness- White = 100; Black = 0), a* (redness; from green to red: positive = red; negative = green), and b* (blueness: from blue to yellow: positive = yellow; negative = blue). [43-45] Color differences ΔE were compared to a white standard tile to

determine colorimeter color values: $\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$. Among red and black sorghum genotypes, ΔE colorimeter values were strongly associated with 3-deoxyanthocyanidin concentrations.[44-46] However, a colorimeter is more suited to evaluating fine particles, so whole grain measurements required many repetitions. Near Infrared Spectroscopy (NIR) analysis uses a larger volume than the colorimeter test. Single panicle samples larger than minimum standards were still useful in the NIR study, meaning fewer observations were needed to identify composition traits.[43, 44]

NIR: near-infrared spectroscopy

Unlike conventional spectroscopy, NIR is a vibrational spectroscopy that uses photon energy (hn) within a primary light beam produced by a source of radiant energy of 2.65×10^{-19} to 7.96×10^{-20} J and discriminated into particular wavelengths (750 to 2,500 nm) to obtain more specific information.[45] It emerged as a technology for food quality control and process monitoring and is becoming progressively more popular among plant breeders, identifying and quantifying all constituents of a sample in a single spectral reading.[46-48] This spectral information is disseminated via chemometrics in conjunction with appropriate wet chemistry reference methods.[48] Following discrimination, the light beam is directed onto the sample, and a photoelectric detector collects and determines the resultant reflected or absorbed radiant energy. The basic relationship between light absorption and the concentration of a solute is described by the Beer-Lambert law $Cx = Ax/e.l$ where

Cx = concentration of the test solute;

Ax = absorption of the test solution;

e = molar absorptivity of the test solute;

l = the path length travelled by the light through the solution.[49]

To meet minimum grain quantities for NIR scans, sorghum panicles were packaged in bulk to create an experimental unit for using near-infrared (NIR) spectroscopy.[50, 51] Infrared light is incident on a solid sample, so it is a variety of NIR with two compelling advantages over conventional methods like colorimetric assays and HPLC: a) NIR could provide rapid and reliable analysis of antioxidant compounds in a wide range of samples, among them, sorghum; [50, 51] b) NIR is a successful analytical method that does not require pre-treating samples so samples are intact and undamaged.[50, 51]

LC-MS

Liquid chromatography-mass spectrometry (LC-MS) is an analytical chemistry technique combining the physical separation capabilities of liquid chromatography (LC) with the mass analysis capabilities of mass spectrometry (MS). Many research studies have been published on HPLC-DAD methods useful to analyze 3-deoxyanthocyanidin from sorghum.[38-41] Sample preparation requires all sorghum samples be ground to achieve an appropriate particle size. Samples were extracted in acidified methanol for approximately two hours in a shaker.[31] The extracts were centrifuged at 3000g for 10 min and then decanted. All extracts were filtered through a 0.45 μm nylon membrane before analysis with HPLC or LC-MS. Although this method is destructive, time-consuming, and costly, it is precise, the most widely used analytical technique for sorghum extractions.[31, 38-41] Chromatographic analyses were done on an HPLC instrument with a diode array detector. A reversed phase C18 column was used for separation. Gradient elution was performed using mobile phase A (5% formic acid aqueous solution) and mobile phase B (methanol) with the flow rate of 1 mL/min. Detection was set at 480 nm for

sorghum 3-deoxyanthocyanidins. Identifying sorghum 3-deoxyanthocyanidins was based on the retention times of commercial standards under UV-vis spectra and LC-MS data. Each 3-deoxyanthocyanidin was quantified by calculating peak areas using a calibration curve for each standard.[16, 31, 38-41] Chemical compounds were also analyzed using mass spectrum (MS). Low-resolution electrospray mass spectrometry was performed (Electrospray ionization=ESI) in negative mode under the following conditions: sheath gas (N_2), 40 units/min; auxiliary gas (N2), 5 units/min; spray voltage 3.5 kV; capillary temperature, 250°C; capillary voltage, 29V; tube lens offset, 60V.[6, 31]

Only a few studies have focused on 3-deoxyanthocyanidin levels in red, black, and purple sorghum extracts.[18, 20, 39, 40, 52] However, considering how little data has been published documenting the phenotypic diversity of anthocyanins in sorghum with virtually nothing known about the relationship between anthocyanins and pericarp colors, identifying phenotypic profile and diversity of anthocyanins in sorghum accessions with different pericarp pigments will help in producing sorghums with optimum levels of desired flavonoids. Sorghum pericarp colors and phenotypic diversity of anthocyanins drove us to investigate the 3-deoxyanthocyanin profile in different accessions using high performance liquid chromatography with diode array detection (HPLC-DAD).

Part 2 Health benefits, structure of anthocyanins in sweetpotato leaves; analytical methods

Background

Sweetpotatoes (*Ipomoea batatas L.*) are seventh among world staple foods after wheat, maize, rice, potato, barley, and cassava.[53, 54] Although both tubers and leaves are edible,

sweetpotato leaves and vines have little economic value in most countries; they are either fed to cattle or used as green manure.[54, 55] Several previous studies have revealed that sweetpotato leaves are not only rich in vitamin B, β-carotene, iron, calcium, zinc, and protein, but are also more tolerant of diseases, pests, and high moisture than other leafy vegetables grown in tropical areas. They can serve as a low-cost source of anthocyanin pigments.[55-61] Tender leaves and stems of sweetpotatoes are the sweetpotato tips or shoots accepted as greens by many people in Asian countries.[56, 57]

Health benefits

Sweetpotato leaves contain several chemical compounds linked to human health. Sweetpotato leaves compare with spinach in nutrient content in several genotypes and under different growing conditions.[56-60] Recent studies show that, compared to the major commercial leafy vegetables like brassicas and lettuce, sweetpotato leaves have more flavonoids, mainly anthocyanins, and phenolic acids that have medicinal value and health benefits.[57] Sweetpotato anthocyanins are also more stable and can be used as natural food colourants.[59] These polyphenolics have many different physiological functions like radical scavenging and are anti-mutagenic, anti-diabetic, and anti-bacterial in *vitro* or in *vivo,* making them superior to many other commercial vegetables.[56, 59-62] The nutritional attributes of sweetpotato leaves are increasingly recognized as a functional food in a western-style diet.

Anthocyanin in sweetpotato leaves

The major pigment in purple-fleshed sweetpotato tubers is acylated anthocyanins, and the chemical structures have been determined by previous studies.[63-66] Purple-fleshed sweetpotatoes

are usually either cyanidin-predominated[63, 64] or peonidin-predominated depending on the ratio of peonidin to cyanidin aglycones (pn/cy). [65, 66] Islam reported that sweetpotato leaves may also serve as a cheap source of anthocyanin among fruits and vegetables, with fifteen total anthocyanins identified in sweetpotato leaves and three unknown constituents under the 520 nm wavelength.[56] The anthocyanins of the sweetpotato leaves were acylated cyanidin and peonidin type with cyanidin-predominating in sweetpotato leaves.[56, 67] Yoshinaga et al. reported cyanidin-predominating pigments are superior to peonidin in antioxidant and anti-mutagenicity activity.[67]

Anthocyanidin content

Previous studies also noted a significant difference ($p<0.0l$) in total anthocyanin content of several types of sweetpotato leaves, ranging from 3.09 to 18.8 CV (color value)/g powder anthocyanin pigments.[56] However, relatively little data have been published about the characteristics of sweetpotato leaves, specifically anthocyanin components or their physiological functions.

Gene expression

AN1 is a regulatory gene that promotes anthocyanin biosynthesis in potato tubers and encodes a transcription factor.[68] D'Amelia et al. demonstrated that *AN1* displays intraspecific sequence variability in both coding/non-coding regions and in the promoter, and its expression is associated with high anthocyanin content in sweetpotato leaves.[68] The classical loci identified for potato leaf anthocyanin accumulation was derived from *AN1*, which indicates it is an important gene expression for the molecular mechanisms underlying anthocyanin biosynthesis in sweetpotato leaves.[68, 69]

Genomic expression has been investigated in other studies, which showed several other anthocyanin biosynthetic genes active in both sweetpotato flesh and leaves: chalcone synthase (*CHS*), chalcone isomerase (*CHI*), dihydroflavonol 4-reductase (*DFR*), flavone 3-hydroxylase(*F3H*), and anthocyanidin synthase (*ANS*).[70] Northern blot analysis of the anthocyanin biosynthetic genes on different parts of mature purple- and yellow-fleshed sweetpotato plants have indicated low level genomic expression of *CHI, F3H, DFR, ANS* in the leaves of both varieties.[71]

Analytical methods

CV densitometer

Sweetpotato leaf powder (0.5g) was extracted in 10 ml of 0.5% H_2SO_4. Leaves were steeped overnight, and the extract was centrifuged and filtered. The resultant supernatant was diluted fourfold with buffer solution, and the pH adjusted to 3.0. The supernatant was used to measure optical densities at 530nm with a dual-wavelength flying spot scanning densitometer with a microplate system. The color value (CV) for the anthocyanin extract, the commercial indicator of total anthocyanin, was calculated using the following formula: $CV=0.1 \times OD_{530} \times 4 \times 20/g$ DW, where OD_{530} is a detected wavelength, 4 and 20 are the dilution levels, and DW is leaf dry weight.[56]

LC

The anthocyanin extracts (0.5g of sweetpotato leaf powder extracted using 10 ml of 0.5% H_2SO_4) were analyzed using an HPLC with a photodiode array detector (DAD) detector. The column used was reversed phase C18 column with column oven at 35°C. The elution solvents were A (1% v/v formic acid in water) and B (acetonitrile containing 1% formic acid) with the

gradient elution at low eluent flow rate. Spectral data from 200 to 600 nm were recorded, and the anthocyanin chromatograms were monitored at 530 nm. Anthocyanin compounds were identified by retention time with references set to commercial standards and UV spectra.[16, 17, 56, 60, 62, 72] However, this procedure for analyzing sweetpotato leaf anthocyanin is for HPLC only. Further structure elucidation of the large, complex anthocyanin compounds requires developing an improved HPLC-MS method.

In conclusion, sweetpotato leaves have widely been neglected, other than use as livestock feed. Using sweetpotato leaves as a vegetable for human consumption could not only significantly increase food availability in many countries with food shortages but also serve as an excellent source of antioxidative polyphenolics to enhance specific bodily functions in addition to being nutritious. Previous studies have only reported on the nutritional composition and physiological functions of the sweetpotato tuber, and the profile and content of phytochemicals like anthocyanins in sweetpotato leaves remain mostly unknown. Moreover, anthocyanin content of vegetation depends heavily on plant growth stages and environmental factors. How anthocyanin content in sweetpotato leaves respond to harvest stages and growth conditions are mostly unknown. For useful information on nutritional value, anthocyanin profiles and content in sweetpotato leaves of different varieties under different growth conditions will not only be useful to plant breeders but also to consumers selecting sweetpotato varieties for anthocyanin health benefits.

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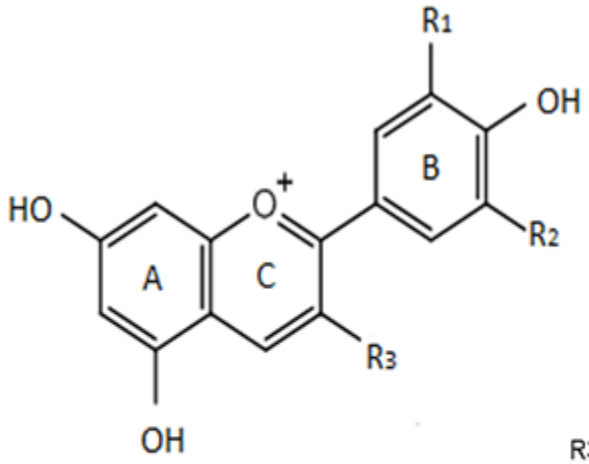
Figure 1-1 Chemical structures of common anthocyanidins and anthocyanins.



	R1	R2
Cyanidin	OH	H
Pelargonidin	H	H
Delphinidin	OH	OH
Petunidin	OCH3	OH
Peonidin	OCH3	H
Malvidin	OCH3	OCH3

R3 = Glucose, galactose, rhamnose, xylose, or arabinose

Figure 1-2 Selected sugars and aromatic or aliphatic acids that commonly occur in anthocyanin structure. Sophorose=2-O-b-D-glucopyranosyl-D-glucose; rutinose=6-O-L-rhamnosyl-D-glucose; sambubiose=β-D-xylosyl-(1-2) - β-D-glucose.[16, 17]

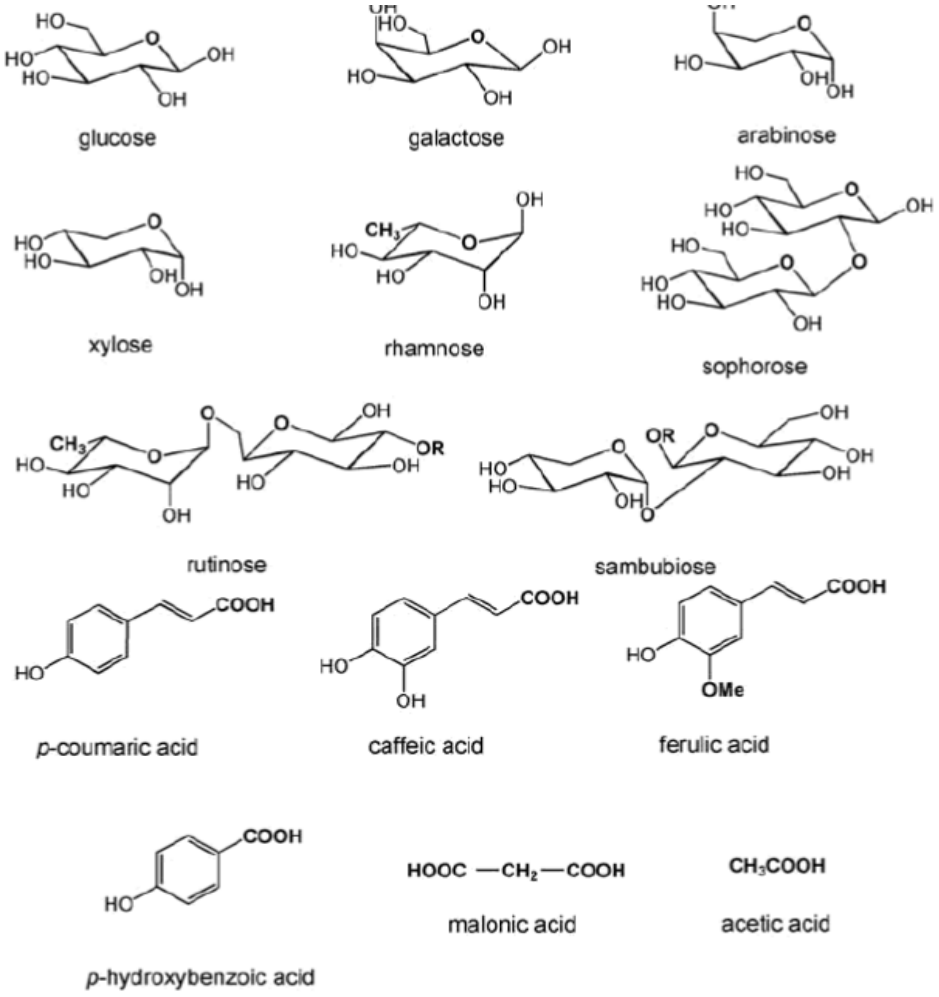


Figure 1-3 Diagram of sorghum caryopsis showing the pericarp, testa, endosperm, and germ.

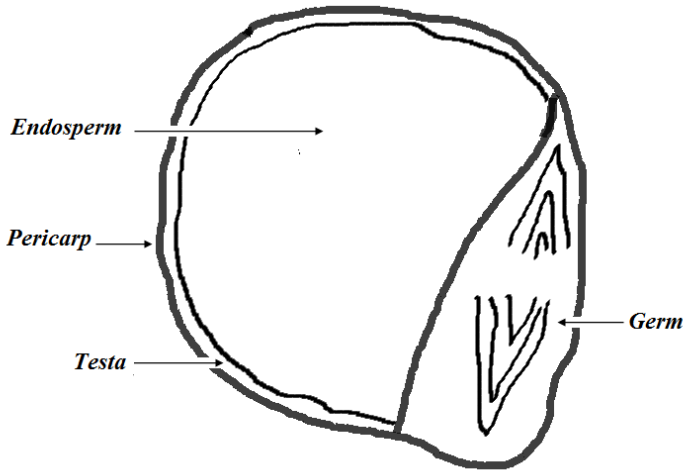
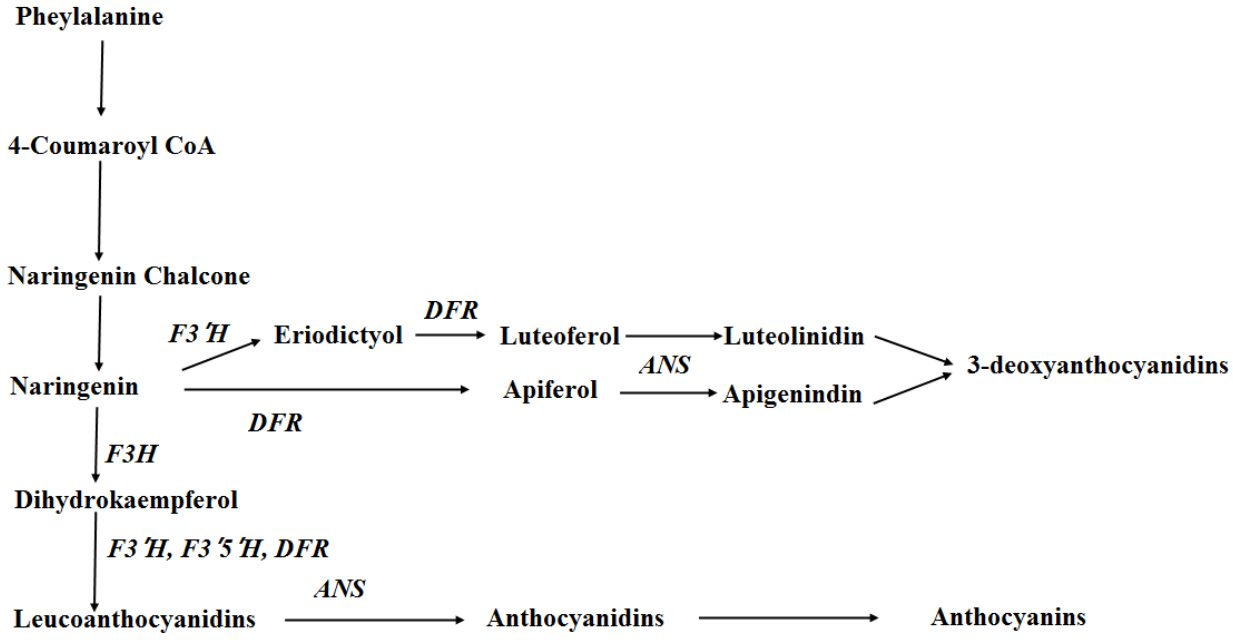


Figure 1-4 Schematic representation of the flavonoid biosynthesis pathway in sorghum. Shown two branches diverging from naringenin and leading to the synthesis of 3-hydroxyflavonoids (anthocyanins) and 3-deoxyanthocyanidins (phytoalexins). Enzymes shown are: F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; DFR, dihydroflavonol reductase and ANS, anthocyanidin synthase.[34, 35]



Chapter 2 - Phenotypic Diversity of Anthocyanins in Sorghum Accessions with Various Pericarp Pigments

Abstract

Anthocyanins, a sub-class of flavonoids, are natural pigments known to have functional health benefits. Sorghum is a rich source of various phytochemicals including anthocyanins. This study was to identify and quantify the profiles of anthocyanins by HPLC-DAD in the selected 25 sorghum accessions with various phenotypic pericarp pigments. The predominant anthocyanins found in sorghums were 3-deoxyanthocyanidins including the unique leuteolinidin and apigeninidin analogs. The high levels of total anthocyanins were found in the red pericarp PI297139 and the brown pericarp PI221723, followed by the brown pericarp PI35038 and the yellow pericarp PI229838. There were moderate to low levels of anthocyanins observed in all the other accessions except for the white pericarp that generally contained least to undetectable amount. Although anthocyanins appeared to be associated with the pericarp color in the sorghum accessions with the highest contents in each pericarp pigment category, a distinguishable diversity of anthocyanin contents was presented among and between the phenotypic pericarp colors, suggesting other colorful phytochemicals such as carotenoids might be contributed. Establishing a database of anthocyanin profile and diversity in sorghum accessions with various pericarp pigments may lead to the development of novel functional sorghum products with active anthocyanin-enriched health benefits.

1. Introduction

Sorghum (Sorghum bicolor) ranks the fifth most staple crop all over the world in term of world grain production [1] and is the mainstay of people in the warmer temperatures and tropical regions of the world such as South Asia and Africa [2]. It is also a good source of proteins, calories and minerals in developing countries [3, 4]. The United States is the largest producer and exporter of sorghum, consisting of 25% of world production and approximately 70% of sorghum export in 2015- 2016 [5].

Among cereals, sorghum contains the highest phytochemical contents with up to 6% (w/w), including anthocyanins, carotenoids, phenolic acids, and condensed tannins, etc. [6-8]. These phytochemicals are widely distributed in the pericarp and testa of the sorghum [9-11]. The color of sorghum pericarp shows a wide range of different colors from red to white. Although both anthocyanins and carotenoids may attribute to the color, the red color of sorghum accessions has been reported due to anthocyanins [12-14]. Furthermore, the pericarp color was considered a dependable indicator for the sorghum varieties and the levels of anthocyanins [12, 15].

Anthocyanins are one of the most important water-soluble plant pigments [16]. They are synthesized by the flavonoid branch of the phenylpropanoid pathway through secondary metabolism in the plants. Among the over 600 types of anthocyanins [17], the majority of anthocyanin aglycone found in the food items usually consists of six anthocyanidins, i.e., cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin [18,19]. However, sorghum anthocyanins are unique 3-deoxyanthocyanidins, which include the orange luteolinidin

and the yellow apigeninidin [20,21]. Figure 2-1 shows the chemical structures of four common sorghum 3-deoxyanthocyanins, i.e., luteolinidin, apigeninidin, 5-methoxyluteolinidin, and 7-methoxyapigeninidin. It appears that sorghum grain is the only known dietary source of 3-deoxyanthocyanidins except for the flowers of sinningia (Sinningia cardinalis), the silk tissues of maize (Zea mays), and the stalks of sugarcane (Saccharum sp.) [22-24]. The exceptional 3-deoxyanthocyanins in sorghum seems more stable than other anthocyanins, making them a desired natural food colorant [9, 15]. While both luteolinidin and apigeninidin analogs were identified in sorghum [21] and the genetic expression of 3-deoxyanthocyanidin synthesis enzymes was investigated [13], the quantitative contents were not reported yet [20]. Furthermore, both luteolinidin and apigeninidin in sorghum belong to phytoalexin due to their responsibilities of anti-fungal invasion and/or anti-stressful activities [13, 25]. Dietary sorghum 3-deoxyanthocyanidins have been also associated with human health benefits such as prevention of obesity and diabetes through antioxidant and anti-inflammatory mechanisms [26].

Considering the relatively little data published documenting the phenotypic diversity of anthocyanins in sorghum and virtually nothing known about the relationship with the pericarp colors, the objective of this study was to identify phenotypic profile and diversity of anthocyanins in the selected 25 sorghum accessions with various pericarp pigments.

2. Material and Methods

2.1. Chemicals

Acetonitrile, methanol, and formic acid at either HPLC grade or analytic grade used in this study were purchased from Thermal Fisher Scientific (Suwanee, GA). Water was purified through Barnstead E-Pure Deionization System (Dubuque, IA) and filtered by Millpore

0.45 μm membrane (Bedford, MA). Commercial standard of Peonidin- 3-glucoside chloride and apigeninidin were purchased from Sigma- Aldrich (St. Louis, MO).

2.2. Sample preparation and extraction

Sample material has been described previously [27]. In brief, accessions were grown in South Carolina in 2012, a subset was selected based on variation on polyphenol content, and bran was obtained by decortication with a tangential abrasive dehulling device. Thresher (Precision Machine Company, Lincoln, NE). Prepared bran was then stored at -80℃ until further extraction. For preparation of anthocyanin extracts, 1 g of the bran was extracted with 10 mL of acidified MeOH with 1N of formic acid at 95:5 (v/v). The flasks containing bran/solvent mixture were wrapped with aluminum foil to avoid light exposure. After a 12-h extraction, the samples were centrifuged at 2,800 rpm for 30 min and then the supernatant was collected and dried by vacuum drier at 25°C overnight. One mL of the acidified MeOH was added and then the dissolved extract was filtered by Whatman syringe filter (Whatman 0.45 μm PVDF) for further HPLC-DAD analysis.

2.3. Identification and quantification of anthocyanins by HPLC-DAD

According to our previous publications [19, 28], Shimadzu HPLC system (Kyoto, Japan) was used for chromatographic analysis and separation. This system employed a DGU-20A3 built in degasser, a LC- 20AB solvent delivery pump, a SIL-20ACHT auto-sampler, a CTO-20 AC column-holding oven, a CBM-20A communicator module, and a SPD-M20A Photodiode Array Detectors. A Waters (Milford, MA) C18 reversed phase column (250 mm length, 4.6 mm diameter) was used for anthocyanin separation. Data was analyzed using LC solution software (Kyoto, Japan). Elution was performed with mobile phase A (5% formic acid in de-ionized water) and mobile phase B (5% formic acid in acetonitrile/water 1:1 v: v). An optimum column

temperature of 25°C was set. At a flow rate of 0.6 mL/min, the gradient conditions were set with solvent B volume as 10-30% for 30 min, 30-55% the following 20 min before returning to 10% at 60 min. The detector performed a full spectrum scan between 190-800 nm, where 480 nm was used for monitoring anthocyanins. Peonidin-3-glucoside was used as an internal standard for estimation of extraction recovery. The contents of anthocyanins were quantitated based upon standard curve of apigeninidin, and the results were expressed as Apigeninidin Equivalent (APGE).

2.4. Statistical analysis

Data were analyzed using SAS statistical software 9.3 (SAS Institute, Cary, NC, USA). Data were analyzed by one-way ANOVA using a general linear model procedure followed by Turkey's post-hoc test. The results were presented as means ± SD, and a probability of $p \leq 0.05$ was considered significant.

3. Results

3.1 HPLC chromatographic separation and identification

Anthocyanin extracts from the representative sorghum accessions were separated by HPLC as shown in Figure 2-2. While anthocyanins were undetectable in the white pericarp sorghum PI656079, a total of four anthocyanins were eluted at the retention times between 16 and 27 min in the yellow PI229838, brown PI 1221723, and red PI 297139. The sorghum anthocyanins or 3-deoxyanthocyanidins were identified based on the retention times of the commercial standards, peak UV–vis spectra, and published LC-MS data [20]. Four major 3-deoxyanthocyanins were identified, i.e., luteolinidin, apigeninidin, 5-methoxyluteolinidin, and 7-methoxyapigeninidin. Their retention times were 17.25, 22.23, 22.92, and 27.77 min,

respectively (Figure 2-2). Both apigeninidin and 7-methoxyapigeninidin were predominant, which counted approximately 60-80% of the total 3-deoxyanthocyanins.

3.2 Quantification of anthocyanin in 25 sorghum accessions

The contents of each anthocyanin and total anthocyanins in 25 sorghum accessions with various pericarp pigments were presented in Table 2-1. Based up the categories of the pericarp colors from red, brown, yellow to white, the high levels of anthocyanins in each category were found in a red pericarp PI297139 (1461.4±98.7 g/kg), followed by two brown pericarp accessions PI221723 and PI35038 (1376.4 ± 33.2, 937.3 ± 29.4 g/kg, respectively) and a yellow pericarp accession PI229838 (574.8 ± 105.4 g/kg). While anthocyanins were undetectable in most of the white sorghum accessions, the moderate to low levels of anthocyanins were observed in all the pericarp pigment categories.

4. Discussion

The objective of the present study was to identify phenotypic diversity of anthocyanins in the selected 25 sorghum accessions with various pericarp pigments that were postulated to be associated with the colored anthocyanins. The profiles of anthocyanins in various categories of the pericarp colors were similar, but the contents varied remarkably. In spite of four 3-deoxyanthocyanin peaks, some unidentified peaks with a similar spectral characteristic of apigeninidin or luteolinidin were revealed. These minor peaks may most likely be derivatives of the 3-deoxyanthocyanidins as suggested by Lafayette [29]. Four 3-deoxyanthocyanins were detected in most of the sorghum accessions in each pigment category except for the white accessions that generally contained least to undetectable anthocyanins. If the contents of anthocyanins were compared among the highest one in each pericarp color category as shown in

Table 2-1, it would appear that the contents of anthocyanins were associated with the pericarp colors clearly. That is to say, the contents of anthocyanins in sorghum accessions seemed to be predicted by the pericarp color. However, a distinguishable diversity of anthocyanin contents was found among and between the phenotypic pericarp colors. In the three red accessions tested, for example, one red accession contained the highest levels of anthocyanins, but the other two had much less levels than the most brown and yellow, even less than one of the white accessions. A high diversity of anthocyanins in sorghum is in agreement with the previous reports by others [9, 11, 30, 31, 32]. Although the capacity of anthocyanin biosynthesis is decided by the genotypic cultivar, the activity of anthocyanin biosynthesis can be actually influenced by many environmental factors.

Furthermore, a number of factors including genotype (cultivar accession) and environment (production practices and ecology, etc.) also determine the phenotypic pigment of a sorghum pericarp. One of the important genotypic factors that may affect the pericarp color is other colored phytochemicals biosynthesized such as carotenoids that may have interfered. Carotenoids are one of the colored phytochemicals that have been suggested to count for the phenotypic color of sorghum pericarp [27]. We conducted a pilot study by detecting carotenoid contents in the nine selected sorghum accessions with various pericarp colors. The highest contents of total carotenoids were found in the sorghum accessions with yellow pericarp, followed by brown pericarp. The lowest carotenoids were observed in the accessions with white pericarp [33]. It appeared that the phenotypic diversity of sorghum pericarp colors might be contributed, at least in part, by the contents of carotenoids.

5. Conclusions

Taken together, the profile of anthocyanins was quantified in the selected 25 sorghum accessions with various phenotypic pericarp pigments. The predominant anthocyanins found in sorghums were 3-deoxyanthocyanidins that were high in one red accession and two brown accessions, followed by a yellow accession. The white accessions contained least to undetectable amount. However, a distinguishable diversity of anthocyanin contents was presented among and between the phenotypic pericarp colors, suggesting other colorful phytochemicals such as carotenoids might have interfered. Future studies by establishing a database of both anthocyanins and carotenoids in sorghum accessions with various pericarp pigments may be warranted.

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Table 2-1 The contents of 3-deoxyanthocyanin in 25 Sorghum Accessions (mg/kg DM APGE) *

Pericarp color	Accessions	Luteolinidin	Apigeninidin	5-Methoxyluteolinidin	7-Methoxyapigeninidin	Total
Red	PI297139	209.1±5.3 [a]	978.8±55.2 [a]	50.8±1.3 [b]	222.8±36.7 [b]	1461.4±98.7 [a]
Red	PI576426	UD	UD	1.0±0.03 [d]	0.8±0.08 [d]	1.7±0.1 [f]
Red	PI329440	UD	0.7±0.02 [f]	UD	UD	0.7±0.02 [f]
Brown	PI221723	144.6±4.1 [c]	888.8±8.7[b]	51.1±4.1 [b]	291.8±16.3 [a]	1376.4±33.2 [b]
Brown	PI35038	162.6±7.9 [b]	552.3±12.2[c]	65.0±1.9 [a]	157.4±7.3 [c]	937.3±29.4 [c]
Brown	PI208537	41.6±0.4 [hg]	33.5±2.3 [ef]	3.0±0.1 [d]	1.5±0.01 [d]	79.6±2.9 [f]
Brown	PI221655	4.7±0.2 [hg]	42.4±0.1[ef]	0.8±0.1 [d]	2.9±0.1 [d]	50.9±0.4 [f]
Brown	PI533957	6.4±0.9 [hg]	36.6±1.5 [ef]	1.0±0.5 [d]	6.5±0.9 [d]	50.5±3.8 [f]
Brown	PI533902	14.0±0.01 [g]	11.7±0.6 [f]	2.3±0.3 [d]	2.5±0.1 [d]	30.5±0.9 [f]
Brown	PI542718	1.8±0.6 [hg]	19.5±3.9 [ef]	1.0±0.2 [d]	2.9±0.2 [d]	25.1±4.9 [f]
Brown	PI656038	11.7±1.7 [hg]	5.2±0.6 [f]	1.2±0.2 [d]	UD	18.1±2.6 [f]
Brown	PI534105	5.4±0.1 [hg]	5.1±1.7 [f]	1.4±0.1 [d]	1.1±0.01 [d]	13.0±1.8 [f]
Brown	PI533792	UD	UD	UD	1.6±0.1 [d]	1.6±0.1 [f]
Brown	PI576425	0.6±0.1[h]	UD	0.7±0.03 [d]	UD	1.3±0.1 [f]
Yellow	PI229838	73.7±14.6 [e]	317.7±55.5 [d]	42.1±8.8 [c]	141.3±26.6 [c]	574.8±105.4 [d]
Yellow	PI221610	86.1±2.8 [d]	71.4±0.4 [e]	2.8±0.1 [d]	3.0±0.01 [d]	163.3±3.3 [e]
Yellow	PI229830	3.0±0.2 [hg]	36.5±1.1 [ef]	1.5±0.01 [d]	2.9±0.3 [d]	43.9±1.7 [f]
Yellow	PI221619	2.6±0.8 [hg]	19.6±0.4 [ef]	UD	1.3±0.2 [d]	23.5±1.4 [f]
Yellow	PI533991	3.7±0.2 [hg]	6.4±0.02 [f]	0.9±0.1 [d]	0.8±0.2 [d]	11.8±0.5 [f]
Yellow	PI229875	2.9±0.5 [hg]	1.3±0.6 [f]	1.0±0.1 [d]	1.5±0.7 [d]	6.6±1.6 [f]
Yellow	PI655978	UD	UD	UD	1.0±0.04 [d]	1.0±0.04 [f]
White	PI656079	3.0±0.2 [hg]	1.1±0.1 [f]	1.4±0.1 [d]	UD	5.5±0.4 [f]
White	PI561072	UD	UD	UD	UD	UD

White	PI656007	UD	UD	UD	UD	UD
White	PI565121	UD	UD	UD	UD	UD

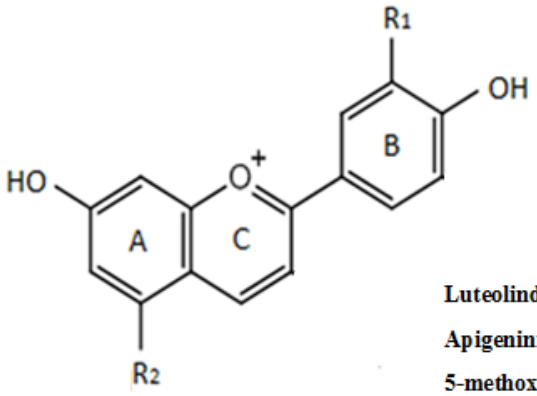
*Values are means ± SD., n=2. Means with different letter within same column differ significantly, $p<0.05$

Figure Legends

Figure 2-1 Chemical structures of anthocyanins identified in sorghum: luteolinidin, apigeninidin, 5-methoxyluteolinidin, and 7-methoxyapigeninidin.

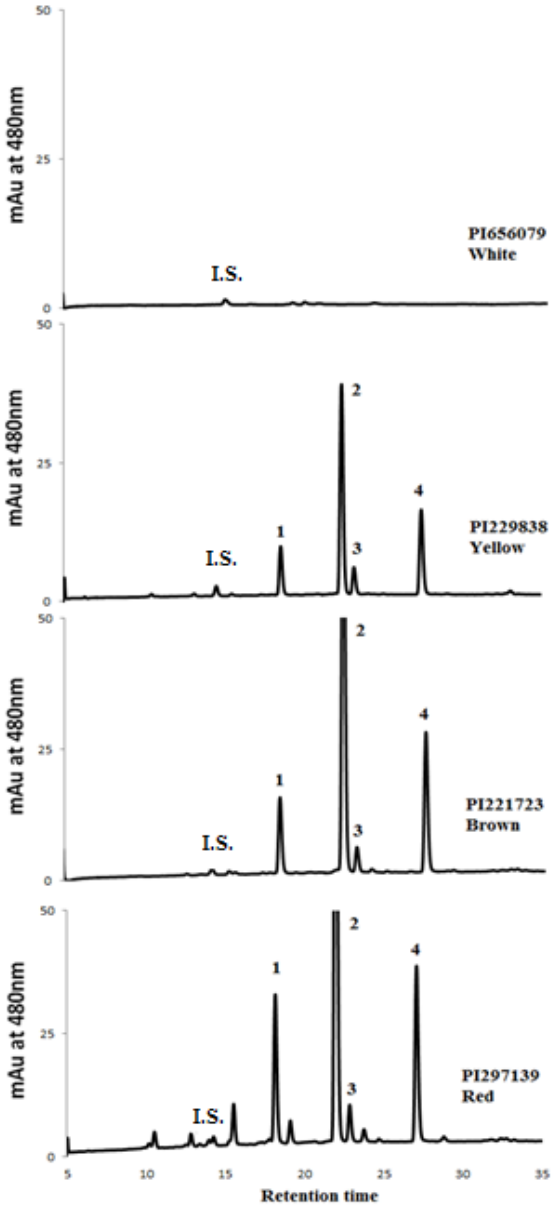
Figure 2-2 Representative HPLC chromatograms of anthocyanins in the selected sorghum accessions with various pericarp pigments: red PI656079, brown PI229838, yellow PI221723, and white PI297139. (Peak 1: Luteolinidin; Peak 2: Apigeninidin; Peak 3: 5-methoxyluteolinidin; Peak 4: 7-methoxyapigeninidin).

Figure 2-1 Chemical structures of anthocyanins identified in sorghum: luteolinidin, apigeninidin, 5-methoxyluteolinidin, and 7-methoxyapigeninidin.



	R1	R2
Luteolindin	OH	OH
Apigeninidin	H	OH
5-methoxyluteolindin	OH	OCH3
5-methoxyapigeninidin	H	OCH3

Figure 2-2 Representative HPLC chromatograms of anthocyanins in the selected sorghum accessions with various pericarp pigments: red PI656079, brown PI229838, yellow PI221723, and white PI297139. (Peak 1: Luteolinidin; Peak 2: Apigeninidin; Peak 3: 5-methoxyluteolinidin; Peak 4: 7-methoxyapigeninidin).



Chapter 3 - Identification and Quantification of Anthocyanins in Purple-fleshed Sweetpotato Leaves

Abstract

As phytochemical-enriched edible greens, sweetpotato (*Ipomoea batatas L.*) leaves have become popular. However, the profile and content of phytochemicals in sweetpotato leaves are mostly unknown. We previously bred a purple-fleshed sweetpotato P40 that demonstrated cancer prevention due to high levels of anthocyanins in the tuberous roots. The objectives of this study were to identify and quantify anthocyanins in P40 leaves when compared with the white-fleshed Bonita and orange-fleshed Beauregard. The mature leaves of P40 at 6-week vine stage were collected and extracted for anthocyanin analysis by HPLC-MS/MS. Fourteen anthocyanins, including a novel anthocyanin (peonidin 3-caffeoyl-p-coumaryl sophoroside-5-glucoside), were identified and quantitated. The contents of anthocyanins in P40 leaves (32.7 ± 2.9 mg/kg DW) were much lower than that in the tubers (13,100 ± 70 mg/kg DW). Furthermore, anthocyanin contents in P40 leaves were even lesser than those of the orange-fleshed Beauregard (334 ± 60.9 mg/kg DW) and white-fleshed Bonita (563 ± 50.4 mg/kg DW). Total phenolic contents as measured by Folin-Ciocalteu were 36.8 ± 4.8 mg GAE/g DW in the leaves of P40, but 41.2 ± 5.0 mg GAE/g DW in Beauregard and 46.7 ± 2.1 mg GAE/g DW in Bonita. No anthocyanin was detectable in the stem of these three sweetpotato varieties. Taken together, this study reports for the first time the profile and content of anthocyanins in the leaves of three sweetpotato varieties with a new anthocyanin identified. The unexpected lower levels of anthocyanins in the purple-fleshed sweetpotato leaves when compared with either the counterpart tuberous roots or the control white-fleshed and orange-fleshed sweetpotato varieties advanced our existing knowledge and also validated a diverse phenotype of anthocyanin biosynthesis between sweetpotato leaves and tubers.

1. Introduction

As the world's sixth most important crop, sweetpotato (*Ipomoea batatas* L.) is a staple food in many developing countries due to high productivity but low input requirements (Bovell-Benjamin, 2007; Claessens, Stoorvogel & Antle, 2008). Sweetpotato is mainly cultivated for tuberous roots combined with huge mass of sweetpotato vines (stems and leaves) after harvesting and 95-98% of the leaves are discarded while the remaining are a potential feedstuff for livestock (Nyaata et al, 2000; Fu et al, 2016). While the tubers of the sweetpotato are nutritious and commonly consumed, sweetpotato leaves are also considered as an edible green in African and Asian countries, which containing protein, essential amino acids, antioxidants, vitamin B, minerals, and dietary fiber (Islam, 2006; Ishiguro et al, 2004; Yoshimoto et al, 2003; Woolfe, 1992). Although sweetpotato leaves are not part of the typical Western diet, sweetpotato green is becoming popular due to a high level of bioactive phytochemicals such as anthocyanins and phenolic acids which possible provides numerous health-promoting benefits (Islam, 2006; Islam 2009; Meyer, Heinonen & Frankel, 2003; Yan-Hwa, Chang, & Hsu, 2000; Wang, Nie, & Zhu, 2016).

Most sweetpotato cultivars have white or yellow flesh, but some have orange flesh that contains carotenoids or purple flesh that contains anthocyanins. Anthocyanins are the predominant subclass of colored flavonoids that consist of red, purple, or blue pigmentations in various plants (Xu & Howard, 2012). Among over 600 types of anthocyanins (Wang & Stoner, 2008), the majority of anthocyanin aglycones found in nature comprise with six anthocyanidins, i.e., cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin with a 2-phenylbenzopyrilium (flavyl-ium) skeleton hydroxylated in 3, 5, and 7 positions by different substitutions at R_1 and R_2 (Figure 3-1). In comparison with other flavonoids, anthocyanins

contain a positive charge on its C-ring leading to different colors in response to various pH (Lim et al, 2013).

Our previous studies demonstrated a purple-fleshed sweetpotato P40 that showed cancer prevention against colorectal cancer due to high levels of anthocyanins in the tuberous roots (Lim et al., 2013; Xu et al, 2015). However, the profile and content of anthocyanins in P40 leaves, although it might be relevant to the tubers, are not fully elucidated. Considering the increasing consumption and the promising health benefits, there is a need to discover and clarify the anthocyanins in the sweetpotato leaves.

To better understand and cover the knowledge gap, the profile and content of anthocyanins in the leaves of the purple-fleshed sweetpotato P40 were identified and quantitated in this study. The results were also compared not only with the counterpart tuberous roots but also with white-fleshed and orange-fleshed sweetpotato varieties. In addition, total phenolic contents in all the three sweetpotato varieties were further exampled.

2. Material and Methods

2.1. Materials

Acetonitrile, methanol, and formic acid used in this study at either HPLC grade or analytic grade were purchased from Thermal Fisher Scientific (Suwanee, GA 30024, USA). Water used was purified through Barnstead E-Pure Deionization System (Dubuque, IA 52001, USA) and filtered by Millpore 0.45 μm membrane (Bedford, MA 01730, USA). A standard of Peonidin-3-glucoside chloride, Folin-Ciocalteu reagent, and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO 63118, USA).

2.2. Sample preparation and anthocyanin extraction

The mature leaves of three sweetpotato varieties (Bonita, Beauregard, and P40) grown in the John C. Pair Horticultural Center, Kansas State University (Haysville, KS 67060, USA) were harvested from the tubers at vine stage for six-weeks of growth cycle. For each variety, leaves were washed with tap water, chopped into approximately 2 cm slices, freeze-dried, and ground by a food processor into powder. The powder was then stored at -80 °C until further extraction.

For preparation of anthocyanin extraction, 0.5 g of the powder was extracted in 20 mL of acidified water with 1 N of formic acid at 95:5 (v/v). The tubes containing powder solvent mixture were wrapped with an aluminum foil to avoid light exposure. After a 24-h extraction, the samples were centrifuged at 4000 rpm for 45 min and then the supernatant was collected and dried by vacuum drier at 25 °C overnight. One mL of the acidified methanol was added and then the dissolved extract was filtered by a Whatman syringe filter at 0.45μm PVDF for next HPLC-MS/MS analysis.

2.3. Identification and analysis of anthocyanins by HPLC-MS/MS

HPLC coupled Electrospray Ionization tandem Mass Spectrometry (HPLC-MS/MS) was used to carry out anthocyanin identification and quantification according to our previous publications (Xu et al., 2015; Su et al., 2016). Briefly, a Shimadzu HPLC system (Kyoto, Japan) was used for chromatographic analysis and separation. This system employed a DGU-20A3 built in degasser, a LC-20AB solvent delivery pump, a SIL-20ACHT auto-sampler, a CTO-20AC column holding oven, a CBM-20A communicator module, and a SPDM20A Photodiode Array Detectors. A Waters (Milford, MA 01757, USA) C18 reversed phase column (250 mm length, 4.6 mm diameter) was used for anthocyanin separation. Data was analyzed using HPLC solution software (Kyoto, Japan). Elution was performed with mobile phase A (5% formic acid in de-

ionized water) and mobile phase B (5% formic acid in acetonitrile/water 1:1 v: v). An optimum column temperature was set at 25 °C. At a flow rate of 0.8 mL/min, the gradient conditions were set as follows: solvent B volume at 20-40% for 30 min, 40-50% in following 5 min and held at 50% for 10 min before returning to 20%. The detector performed a full spectrum scan between 190 and 800 nm, where 520 nm was used for monitoring anthocyanins. Peonidin-3-glucoside was used as an internal standard for quantitation of extraction recovery, and the anthocyanin contents were expressed as peonidin 3-glucoside equivalent (PN3GE) per g dry weight (DW). Based on a signal-to-noise ratio of 3:1 and the standard deviation of the lowest concentration of PN3G/slope of the calibration line, the detection limit was estimated to be 5 µmol.

Mass spectrometric scan was performed on a Bruker Esquire 3000 in positive mode with a scanning interval 500-1200 m/z. Nebulization was conducted at 350 °C aided by concurrent N_2 flow at 10 psi; capillary and cone voltages were set at 3.5 kV and 40 V; drying gas flow rate was 5 L/min. Mass of precursor ions and reactions of fragments loss were evaluated. Data were analyzed using Bruker Hystar Post Processing software (Bruker Daltonics, GmbH, Billerica, MA 01821, USA). The ESI/MS data was used to confirm the mass of each anthocyanin HPLC peak. The mass spectrometry instrument was controlled by the esquire control 5.3 software (Bruker Daltonics, GmbH, Billerica, MA 01821, USA) and the data were processed with Data analysis 3.3 software (Bruker Daltonics, GmbH, Billerica, MA 01821, USA). Identification of each anthocyanin was accomplished by comparison of HPLC retention time, absorbance spectra, and MS spectra with our previously published database (Lim et al., 2013; Xu et al., 2015; Su et al. 2016) and the National Institute of Standards and Technology Mass Spectra Library data (NIST08, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA).

2.4. Total phenolic content

Total phenolics in the extract of each sweetpotato variety were measured by Folin-Ciocalteu method according to the published method by Singleton & Rossi (1965) with a slight modification. Briefly, a stock solution of gallic acid at 1 mg/mL in distilled water was prepared. Then the stock solution was diluted to 12.5-200 µg/mL in 70% acetone for a standard curve. To each of the 96 wells, 75 µl distilled water was added, followed by 25 µl either aliquot of extract or various concentrations of gallic acid solution. Folin-Ciocalteu regent diluted by 1:1 with distilled water was then added to each well. The reaction was then allowed to stand for 10 min at room temperature, and then 100 µl of Na_2CO_3 solution at 7.5% (w/v) was added to each well. Plate was covered and kept in dark for 90 min before measuring. Absorbance were read in a microplate reader Synergy HT (BioTek Inc., Winnoski, VT 05404, USA) with Gen5^{TM}2.0 data analysis software. Result were expressed as mg gallic acid equivalent (GAE) per g DW.

2.5. Statistical analysis

Data were analyzed using SAS statistical software version 9.3 (SAS Institute, Cary, NC 27511, USA). Results were evaluated by one way ANOVA using a general linear model procedure followed by Tukey's post-hoc test. The results were presented as means ± SD, and a probability at $p \leq 0.05$ was considered significant.

3. Results and discussion

3.1. Chromatographic separation

The objectives of this study were focused on characterizing the anthocyanin profile and quantifying the anthocyanin contents in each varieties of the sweetpotato leaves using HPLC-MS/MS. The profile of the anthocyanin peaks from sweetpotato leaves versus counterpart tubers

were shown by representative HPLC chromatograms in Figure 3-2. In addition to the internal standard, a total of fourteen peaks were eluted and detected at the retention times between 3.61 and 26.09 min in the leaves or tubers of three sweetpotato varieties except for the tubers of Bonita and Beauregard. Of these, peaks 8 and 9 appeared to be the major anthocyanins and their peak areas were more than half of the total anthocyanin peak areas. No any anthocyanin peaks were detectable in the extract of sweetpotato stems in all of the three varieties (data not shown).

3.2. Mass spectrometric identification

Following HPLC separation, HPLC-MS/MS data were characterized by monitoring the molecular ion characteristics for each peak. The *m*/*z* ratio of each intact anthocyanin and its fragment ions are listed in Table 3-1.

As shown in Table 3-1, cyanidin (*m*/*z* 287) and peonidin (*m*/*z* 301) were the two anthocyanidins only detected in the three sweetpotato varieties. Thirteen of the fourteen anthocyanins including cyanidins (peaks 1, 3, 4, 7-10) and peonidin (peaks 2, 5, 6, 11-13) have been reported previously (Islam et al., 2002a,b; Lee et al., 2013), but one peonidin (peaks 14) was newly found in the leaves of three sweetpotato varieties for the first time. As shown in Figure 3-3, the m/z ratio of molecular ion with fragment ion for peak 4 was captured as an intact molecular of peonidin 3-caffeoyl-p-coumaryl sophoroside-5-glucoside (*m*/*z* 1095) and three major fragments of peonidin 3-caffeoyl-p-coumaryl sophoroside (*m*/*z* 933), peonidin-5-glucoside (*m*/*z* 463), and peonidin (*m*/*z* 301). Transitions from *m*/*z* 1095 to 933 and *m*/*z* 1095 to 463 implied the loss of glucose (*m*/*z* 162) and 3-caffeoyl-p-coumaryl sophoroside (*m*/*z* 632),

respectively. Transition from *m*/*z* 1095 to 301 produced peonidin (*m*/*z* 301) by the loss of glucose and 3-caffeoyl-p-coumaryl sophoroside.

A new anthocyanin detected in the sweetpotato leaves might be attributed by the effective extraction and improved HPLC method, which allowed for a distinct peak separation. When compared with our previous studies (Xu et al., 2013; Su et al., 2016), the composition of mobile phase and the flow rate have been adjusted in this study for better peak separation. Moreover, 5% of formic acid has been added to the water before extraction, creating a low pH environment for anthocyanin stabilization as suggested by Sang et al. (2017) that the peak area would be increased and the detection would be more sensitive under acid condition due to the increased percentage of the flavylium cation.

3.3. Anthocyanin contents in sweetpotato leaves

Table 3-2 lists the contents of individual and total anthocyanins in the leaves and tubers of three sweetpotato varieties. The predominant two anthocyanins, i.e., cyanidin 3-(6,6'-dicaffeoyl-sophoroside)-5-glucoside and cyanidin3-(6,6'-caffeoyl-p-hydroxybenzoyl sophoroside)-5-glucoside, comprised up to more than half of total anthocyanins, while cyanidin 3-p-hydroxybenzoyl sophoroside-5-glucoside exhibited the highest level in both leaves and tubers of P40. The newly identified anthocyanin (peonidin 3-caffeoyl-p-coumaryl sophoroside-5-glucoside) made up approximately 1% of the total anthocyanins.

It should be noted that the total contents of anthocyanins in P40 leaves (32.7 ± 2.9 mg/kg DW) were much lower than that in the tubers (13,100 ± 70 mg/kg DW), implying that the genotype of anthocyanin biosynthesis should be exceedingly diverse between the leaves and

tubers. This is in agreement with the report by Mano et al. (2007) that one member of transcriptional factors, i.e., *MYB*, that was sufficient for induction of all structural anthocyanin genes, was predominantly expressed in the purple-flesh tubers but not in other related-tissues such as stems, leaves, and flowers. Mano et al. (2007) also reported *MYB* was only expressed in the tubers of purple-fleshed sweetpotatoes but not in the tubers of orange-, yellow-, or white-fleshed varieties. This supports our finding which no datable anthocyanins were found in the tubers of orange-fleshed Beauregard and white-fleshed Bonita. It is unexpected that the total anthocyanin contents in P40 leaves were even lesser than those of the orange-fleshed Beauregard and white-fleshed Bonita. A future study by confirming the genotype of anthocyanin biosynthesis in response to different phenotype among different sweetpotato varieties may be warranted.

3.4. Total phenolic content

Total phenolic contents as measured by Folin-Ciocalteu were 36.8 ± 4.8 mg GAE/g DW in the leaves of P40, but 41.2 ± 5.0 mg GAE/g DW in Beauregard and 46.7 ± 2.1 mg GAE/g DW in Bonita (Table 3-3). Significant lower levels of total phenolics in purple-fleshed P40 leaves when compared with orange-fleshed Beauregard and white-fleshed Bonita may suggest anthocyanins might be counted as a majority of phenolics in addition to other minor flavonoids and phenolic acids, etc.

4. Conclusions

In conclusion, this study reports for the first time the profile and content of anthocyanins in the leaves of three sweetpotato varieties with a new anthocyanin identified. The unexpected

lower levels of anthocyanins in the purple-fleshed sweetpotato leaves when compared with either the counterpart tuberous roots or the control white-fleshed and orange-fleshed sweetpotato varieties advanced our existing knowledge and also validated a diverse phenotype of anthocyanin biosynthesis between sweetpotato leaves and tubers.

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Table 3-1 Mass Spectrometric Data of Anthocyanins in the Leaves of Purple-fleshed Sweetpotato P40, orange-fleshed Beauregard, or white-fleshed Bonita

Peak #	Retention (min)	Anthocyanins	[M+H] (m/z)	Fragment ions(m/z)
1	3.61	Cyanidin 3-sophoroside-5-glucoside	773	661,449,287
2	5.28	Peonidin 3-sophoroside-5-glucoside	787	595,433,271
3	6.23	*p*-hydroxybenzoylated (Cyanidin 3-sophoreside-5-glucoside)	893	731,449,287
4	6.96	Caffeoylated (Cyanidin 3-sophoroside-5-glucoside)	935	773,449,287
5	9.75	*p*-hydroxybenzoylated (Peonidin 3-sophoroside-5-glucoside)	907	745,463,301
6	10.29	Caffeoylated (Peonidin 3-sopheroside-5-glucoside)	949	787,463,301
7	11.31	Feruloylated (Cyanidin 3-sophoroside-5-glucoside)	949	787,449,287
8	17.90	Cyanidin 3-(6,6'-dicaffeoyl sophoroside)-5-glucoside	1097	935,449,287
9	17.57	Cyanidin 3-(6,6'-caffeoyl-p-hydroxybenzoyl sophoroside)-5-glucoside	1055	893,449,287
10	21.26	Cyanidin 3-(6,6'-caffeoyl-feruloyl-sophoroside)-5-glucoside	1111	949,449,287
11	22.23	Peonidin 3-(6,6'-dicaffeoyl-sophoroside)-5-glueoside	1111	949,463,301
12	22.85	Peonidin 3-(6,6'-caffeoyl-p-hydroxybenzoyl sophoreside)-5-glucoside	1069	907,463,301
13	25.30	Peonidin 3-(6,6'-caffeoyl-feruloyl sophoroside)-5-glucoside	1125	963,463,301
14	26.09	Peonidin 3-caffeoyl-p-coumaryl sophoroside-5-glucoside	1095	933,463,301

Table 3-2 The contents of individual and total anthocyanins in the leaves and tubers of purple-fleshed sweetpotato P40, orange-fleshed Beauregard, and white-fleshed Bonita (mg PN3GE/kg DM)*

Anthocyanins	P40		Beauregard		Bonita	
	Leaves	tubers	Leaves	tubers	Leaves	tubers
Cyanidin 3-sophoroside-5-glucoside	3.8±0.7^{a}	312.1±19.8^{c}	6.0±2.8^{b}	UD	9.4±1.0^{b}	UD
Peonidin 3-sophoroside-5-glucoside	0.8±0.1^{a}	52.9±0.8^{d}	1.7±0.3^{b}	UD	4.9±2.3^{c}	UD
p-hydroxybenzoylated (Cyanidin 3-sophoreside-5-glucoside)	2.7±0.1^{a}	604.6±51.7^{d}	7.6±1.4^{b}	UD	24.4±2.4^{c}	UD
Caffeoylated (Cyanidin 3-sophoroside-5-glucoside)	3.3±0.4^{a}	180.1±11.9^{d}	30.9±8.8^{b}	UD	48.0±3.9^{c}	UD
p-hydroxybenzoylated (Peonidin 3-sophoroside-5-glucoside)	1.0±0.1^{a}	132.8±5.0^{d}	1.6±0.3^{b}	UD	7.4±0.5^{c}	UD
Caffeoylated (Peonidin 3-sopheroside-5-glucoside)	1.0±0.1^{a}	46.6±0.6^{d}	8.7±2.2^{b}	UD	23.5±0.9^{c}	UD
Feruloylated (Cyanidin 3-sophoroside-5-glucoside)	1.1±0.1^{a}	297.0±12.6^{d}	3.8±0.7^{b}	UD	6.6±1.1^{c}	UD
Cyanidin 3-(6,6'-dicaffeoyl-sophoroside)-5-glucoside	11.6±1.3^{a}	1481.4±18.4^{c}	82.1±15.4^{b}	UD	86.1±7.6^{b}	UD
Cyanidin 3-(6,6'-caffeoylphydroxybenzoyl sophoroside)-5-glucoside	9.8±1.3^{a}	5667.9±34.1^{d}	37.7±6.3^{b}	UD	65.8±15.3^{c}	UD
Cyanidin 3-(6,6'-caffeoylferuloylsophoroside)-5-glucoside	1.9±0.4^{a}	1877.3±19.0^{d}	8.6±2.2^{b}	UD	11.7±0.8^{c}	UD
Peonidin 3-(6,6'-dicaffeoylsophoroside)-5-glueoside	2.3±0.6^{a}	381.6±9.2^{d}	30±7.8^{b}	UD	55.4±4.2^{c}	UD
Peonidin 3-(6,6'-caffeoylphydroxybenzoyl sophoreside)-5-glucoside	1.3±0.1^{a}	1620.9±9.1^{d}	8.9±1.9^{b}	UD	24.5±3.1^{c}	UD
Peonidin 3-(6,6'-caffeoylferuloylsophoroside)-5-glucoside	1.0±0.1^{a}	344.3±7.1^{d}	4.4±1.4^{b}	UD	7.1±1.2^{c}	UD
Peonidin 3-caffeoyl-p-coumaryl sophoroside-5-glucoside	1.1±0.2^{a}	59.3±8.5^{d}	2.8±0.8^{b}	UD	5.4±0.3^{c}	UD
Total Anthocyanins	38±2.9^{a}	13100±70^{d}	240±60.9^{b}	UD	448±50.4^{c}	UD

***** Data are expressed as mean ± SD (n = 3). Values marked by different letters within same row indicate significant difference ($p < 0.05$); UD: Undetectable.

Table 3-3 Total phenolic contents in the leaves of sweetpotatoes (mg GAE/g DW)*

P40	Beauregard	Bonita
36.8 ± 4.8^{a}	41.2 ± 5.0^{b}	46.7 ± 2.1^{c}

* Data are expressed as mean ± SD (n = 3). Values marked by different letters indicate significant difference ($p < 0.05$).

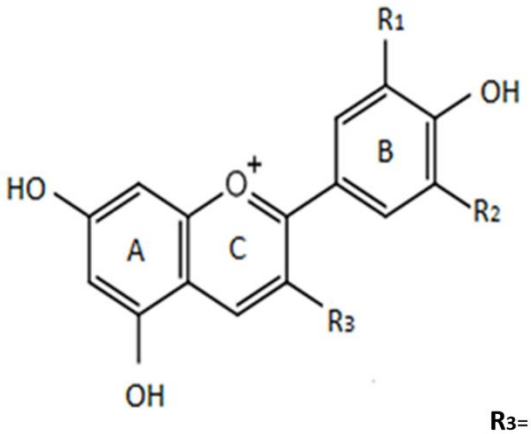
Figure Legends

Figure 3-1 Chemical structures of common anthocyanidins and anthocyanins.

Figure 3-2 Representative HPLC chromatograms of anthocyanins in sweetpotato leave (left) versus tubers (right). The peak number corresponding to each anthocyanin is shown in Table 1.

Figure 3-3 Mass spectra data of peak 14 as a new anthocyanin identified in sweetpotato leaves: (Top Panel) molecular ion spectra of peonidin 3-caffeoyl-p-coumaryl sophoroside-5-glucoside; (Bottom Panel) fragment ion spectra of peonidin, peonidin-5-glucoside, and peonidin-3-caffeoyl-p-coumaryl sophoroside, respectively.

Figure 3-1 Chemical structures of common anthocyanidins and anthocyanins.



	R1	R2
Cyanidin	OH	H
Pelargonidin	H	H
Delphinidin	OH	OH
Petunidin	OCH3	OH
Peonidin	OCH3	H
Malvidin	OCH3	OCH3

R3= Glucose, Galactose, Rhamnose, Xylose, or Arabinose

Figure 3-2 Representative HPLC chromatograms of anthocyanins in sweetpotato leave (left) versus tubers (right). The peak number corresponding to each anthocyanin is shown in Table 1.

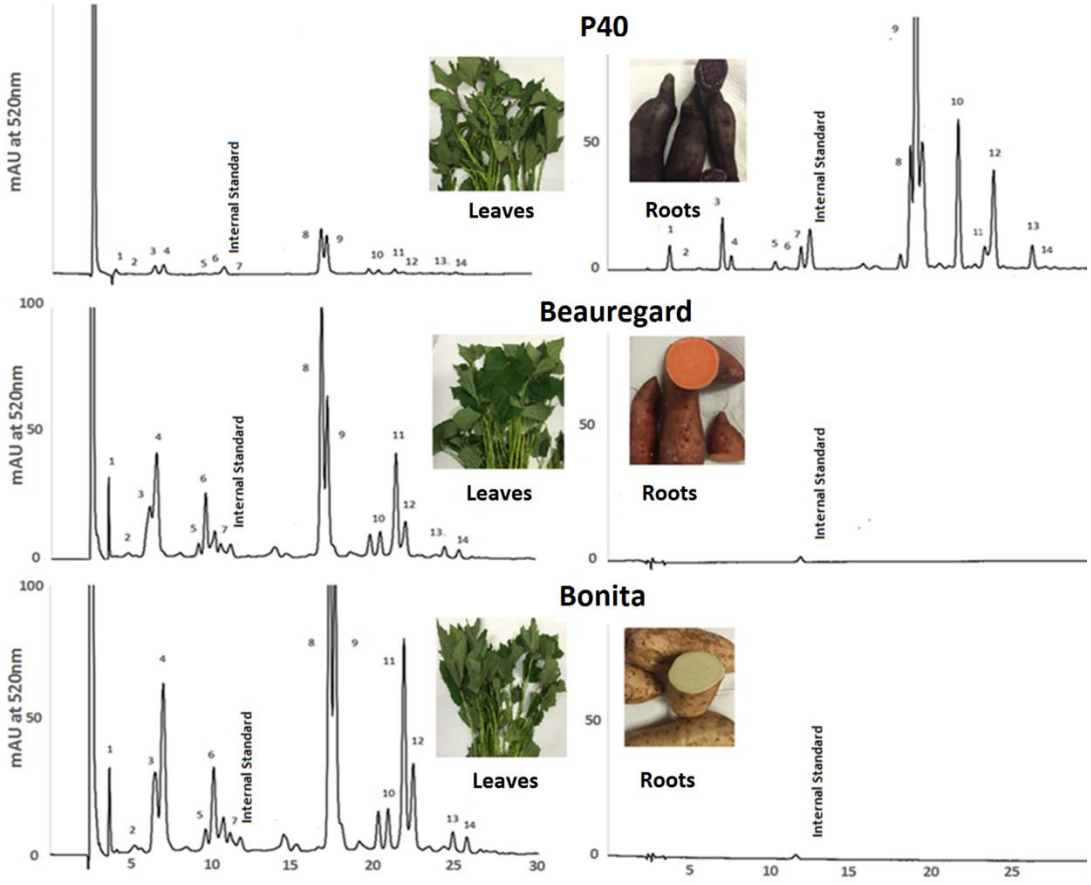
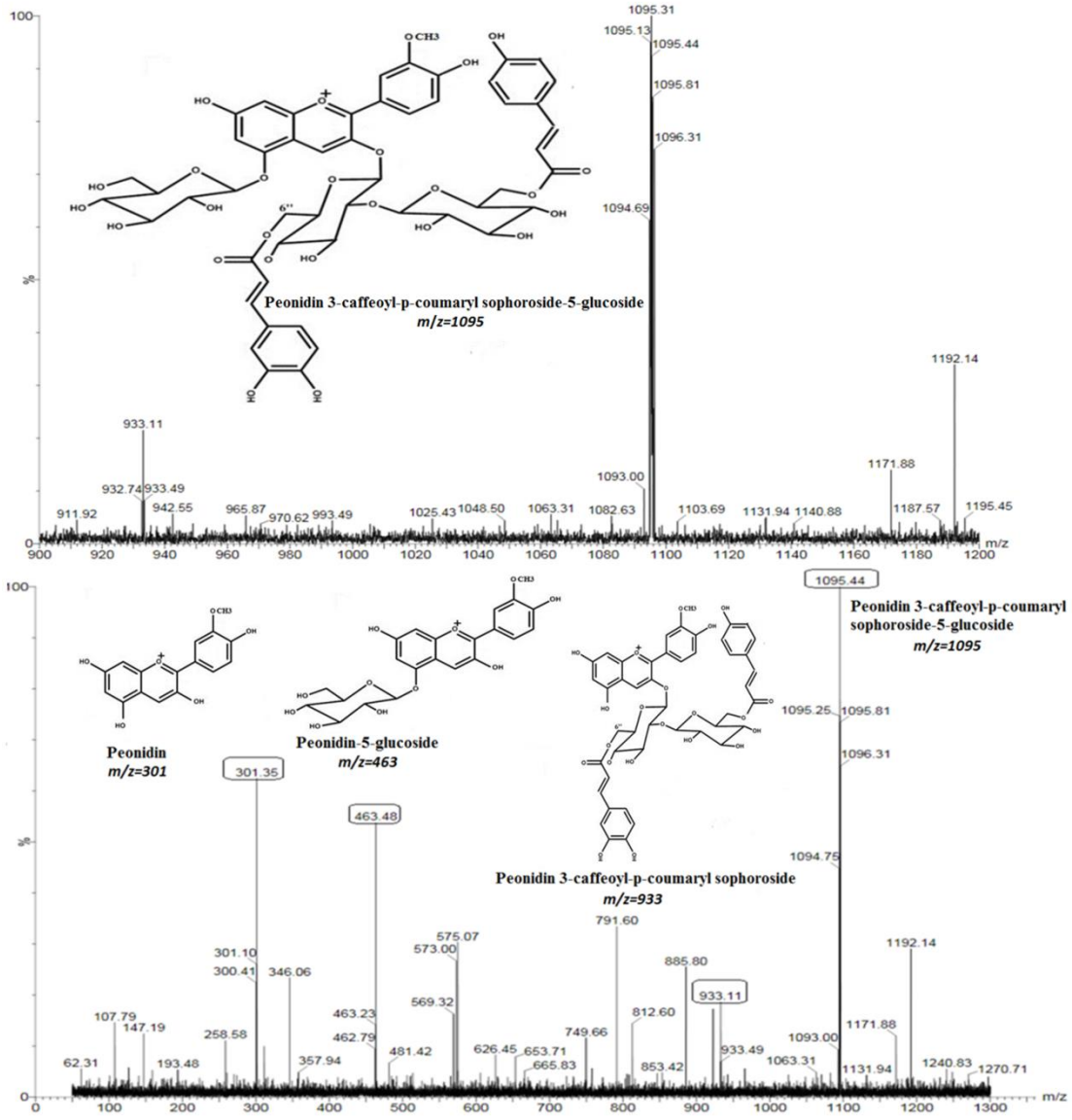


Figure 3-3 Mass spectra data of peak 14 as a new anthocyanin identified in sweetpotato leaves: (Top Panel) molecular ion spectra of peonidin 3-caffeoyl-p-coumaryl sophoroside-5-glucoside; (Bottom Panel) fragment ion spectra of peonidin, peonidin-5-glucoside, and peonidin-3-caffeoyl-p-coumaryl sophoroside, respectively.



Chapter 4 - Characterization of Anthocyanins in Sweetpotato leaves in response to Various Growth Stages and Conditions

Abstract

As phytochemical-enriched edible greens, sweetpotato (Ipomoea batatas L.) leaves have potential health benefits. However, how anthocyanin content in sweetpotato leaves responds to harvest stages and growth conditions remains mostly unknown. In this study, we investigated the effect of harvest timing on the accumulation of anthocyanin in the leaves of several sweetpotato varieties: white-skinned and white-fleshed Bonita, red-skinned and orange-fleshed Beauregard, red-skinned and white-fleshed Murasaki, and purple-skinned and purple-fleshed P40. Anthocyanin content increased continuously in Bonita from 1st slip stage to vine stage, but P40 did not have the same response. Beauregard had most anthocyanin (592.5 ± 86.4 mg /kg DW) and total phenolic content (52.2 ± 3 mg GAE/g DW) of mature leaves at vine stage. The P40 variety had low anthocyanin and total phenolic content in the leaves although P40 tubers have the highest among these varieties. In the high tunnel studies, no significant differences in anthocyanin content were found in Beauregard leaves grown in the high tunnels versus the open field. Our study showed for the first time that anthocyanin levels were significantly affected by the growth stages. Our overall results indicate that growth stage and/or environmental factors among sweetpotato varieties affected anthocyanin content, which is highly variable and genotype-dependent.

1. Introduction

Sweetpotato (*Ipomoea batatas* L.), one of the six most important food crops in the world, is cultivated in many developing countries.[1] This well-known crop is highly productive although it requires low input.[2] The tubers of the sweetpotato are nutritious and commonly consumed, but sweetpotato generates many by-products like leaves, residue, and waste water. Sweetpotato leaves, the above ground parts of the sweetpotato, have been consumed as a green leaf vegetable in African and Asian countries and contain protein, essential amino acids, antioxidants, vitamin B, minerals, and dietary fiber.[3-6] Several authors have reported that both purple and green sweetpotato leaves had high levels of phytochemicals and other bioactive compounds that are excellent sources of anti-oxidative polyphenols like anthocyanins and phenolic acids.[7-9] Recent studies note that these anthocyanins and phenolic acids[10] scavenge free radicals and are anti-mutagenic, anticancer, and anti-bacterial.[3]

Anthocyanins are the primary subclass of polyphenols in the red, purple, and blue pigmentation of many plants. They represent a diverse group of secondary metabolites in higher plants.[11] Among the more than 600 types of anthocyanin, [12] most anthocyanin aglycones found in nature comprise six anthocyanidins: cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin, each with a 2-phenylbenzopyrilium (flavyl-ium) skeleton hydroxylated in the 3, 5, and 7 positions, with different substitutions at R_1 and R_2 (see Figure 4-1). Unlike other flavonoids, anthocyanins have a positive charge on the C-ring, which responds to pH with different colors.[13] Islam et al.[3] demonstrated that sweetpotato leaves are an excellent source of anti-oxidative polyphenols like anthocyanins and phenolic acids, which are a functional food ingredient that enhances the antioxidant capacity of consumed food products in the U.S. food market.

Previous animal studies have demonstrated that anthocyanin-rich purple sweetpotato (P40) may protect against colorectal cancer by inducing cell-cycle arrest and anti-proliferative and apoptotic mechanisms.[13, 14] Furthermore, previous research studies have reported sweetpotato leaves have different anthocyanin content (AC) and total phenolic content (TPC), validating the different phenotypes for anthocyanin biosynthesis in sweetpotato leaves and tubers.

Phytochemical content depends not only on genotype but also on environment. Fluctuations in light, water stress, temperature, rain intensity, and air humidity restrict nutritive components and could also affect plant phytochemical content and quality.[15-17] For example, high tunnel growth chambers provide an easy, cost-effective way to maintain stable growth conditions and extend the sweetpotato growth season, which enhances yield and profitability. Growth stage is yet another source of variability that influences secondary metabolite concentration.[18, 19] Therefore, determining optimum growth stages and growth conditions for sweetpotato leaves should maximize phytochemical content (AC and TPC) and help in assessing sweetpotato leaves as feasible, functional food.[20]

Four leafy varieties (*Ipomoea batatas L.*) of sweetpotatoes (Bonita, Beauregard, Murasaki, and P40) were compared to characterize anthocyanin and total phenolic content at the slip and vine stages. The 1st sweetpotato slips were removed from tubers at two weeks of the growth cycle, and the 2nd sweetpotato slips at six weeks. The vine stage came from 2nd sweetpotato slips that were cut and re-planted for an additional six weeks of growth.

To our best knowledge, no report has been published on the differences in AC and TPC at different sweetpotato leaf development stages. Few studies have been conducted on how high tunnel and open field growth conditions affect AC and TPC of sweetpotato leaves. This study

was set up to investigate the effect of various growth stages and conditions on anthocyanin accumulation in the leaves of four sweetpotato varieties: white-skin and white-fleshed Bonita, red-skin and orange-fleshed Beauregard, red-skin and white-fleshed Murasaki, and purple-skin and purple-fleshed P40. We anticipated the results would provide anthocyanin profiles and content in sweetpotato leaves of different varieties at varying growth stages and conditions.

2. Materials and Methods

2.1. Materials

Acetonitrile, methanol (MeOH), and formic acid used in this study was either HPLC grade or analytic grade and were purchased from Thermal Fisher Scientific (Suwanee, GA, USA). Water was purified through Barnstead E-Pure Deionization System (Dubuque, IA, USA) and filtered through Millpore 0.45 μm membrane (Bedford, MA, USA). A standard of Peonidin-3-glucoside chloride was purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin-Ciocalteu reagent and gallic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Sample preparation and extraction

The leaves of four sweetpotato cultivars (Bonita, Beauregard, Murasaki, and P40) were harvested at the Kansas State University, John C. Pair Horticultural Center. For each variety, leaves were washed with tap water, chopped into approximately 2 cm slices, freeze-dried (Labconco, FreeZone 2.5), and ground by a food processor into powder. Prepared powder was then stored at -80°C until further extraction. To prepare anthocyanin extracts, 0.5 g of powder was extracted with 20 mL of acidified water and 1 N formic acid at 95:5 (v/v). Tubes containing powder/solvent mixture were wrapped with aluminum foil to avoid light exposure. After a 24-h extraction, the samples were centrifuged at 4000 rpm for 45 min, and the supernatant was

collected and dried by vacuum drier at 25°C overnight. One milliliter of acidified MeOH was added, and then the dissolved extract was filtered through a Whatman syringe filter (Whatman 0.45µm PVDF) for LC-MS/MS analysis.

2.3. Identification and analysis of anthocyanins by LC-MS/MS

LC coupled Electrospray Ionization tandem Mass Spectrometry (LC-MS/MS) was used to carry out anthocyanin identification and quantification. A Shimadzu HPLC system (Kyoto, Japan) was used for chromatographic analysis and separation. This system used a DGU-20A3 built-in degasser, a LC-20AB solvent delivery pump, a SIL-20ACHT auto-sampler, a CTO-20AC column holding oven, a CBM-20A communicator module, and a SPDM20A Photodiode Array Detector. A Waters (Milford, MA, USA) C18 reversed phase column (250 mm length, 4.6 mm diameter) was used for anthocyanin separation. Data was analyzed using LC solution software (Kyoto, Japan). Elution was performed with mobile phase A (5% formic acid in de-ionized water) and mobile phase B (5% formic acid in acetonitrile/water 1:1 v:v). An optimum column temperature was set at 25°C. At a flow rate of 0.8 mL/min, the gradient conditions were set as follows: solvent B volume at 20-40% for 30 min, 40-50% for the following five min, held at 50% for 10 min, before returning to 20%. The detector performed a full spectrum scan between 190 and 800 nm, with 520 nm was used for monitoring anthocyanins. Peonidin-3-glucoside was used as an internal standard for quantitation of extraction recovery, and the anthocyanin contents were expressed as peonidin 3-glucoside equivalent (PN3GE) per g dry weight (DW). Based on a signal-to-noise ratio of 3:1 and the standard deviation of the lowest concentration of PN3G/slope of the calibration line, the detection limit was estimated at 5 µmol.

Mass spectrometric scan was performed on a Bruker Esquire 3000 in positive mode with a scanning interval 500-1200 m/z. Nebulization was conducted at 350℃ aided by concurrent N_2

flow at 10 psi; capillary and cone voltages were set at 3.5 kV and 40 V; drying gas flow rate was 5 L/min. Mass of precursor ions and reactions of fragments loss were evaluated. Data were analyzed using Bruker Hystar Post Processing software (Bruker Daltonics, GmbH, Billerica, MA, USA). The ESI/MS data was used to confirm the mass of each anthocyanin HPLC peak. The mass spectrometry instrument was controlled by the esquire control 5.3 software (Bruker Daltonics, GmbH, Billerica, MA, USA), and the data were processed with Data analysis 3.3 software (Bruker Daltonics, GmbH, Billerica, MA, USA). Each anthocyanin was individually identified by comparing HPLC retention time, absorbance spectra, and MS spectra with our previously published anthocyanin data[7-9, 13]. The new anthocyanins were identified by matching mass spectral data with those from the National Institute of Standards and Technology Mass Spectra Library data (NIST08, National Institute of Standards and Technology, Gaithersburg, MD, USA).

2.4. Total phenolic content

Total phenolics in each sweetpotato extract were measured by Folin-Ciocalteu method (Singleton & Rossi, 1965) with a slight modification. A stock solution of 1mg/mL Gallic acid in distilled water was prepared ranging from 12.5-200 µg/mL in 70% acetone for the standard curve. A solution of Na_2CO_3 at 7.5% (w/v) was also prepared. To each of the 96 wells, 75µl distilled water was added, followed by 25µl of either aliquots of extracts or gallic acid standard at various concentrations. Folin-Ciocalteu regent diluted 1:1 with distilled water was then added to each well. The wells then stood for 10 min at room temperature before 100µl of Na_2CO_3 solution was added to each well. Plate was covered and stood in darkness for 90min before measuring. Absorbance was read in a microplate reader Synergy HT, biotek (winnoski, USA)

using Gen5^{TM}2.0 data analysis software. Results were expressed as mg Gallic acid equivalent (GAE) per g dry weight (DW)

2.5. Statistical analysis

Data were analyzed using SAS statistical software, version 9.3 (SAS Institute, Cary, NC, USA). Data were analyzed by overall two-way ANOVA followed by Tukey's test for individual between-group comparisons. The results were presented as means ± SD, and a probability at $p \leq 0.05$ was considered significant.

3. Results and discussion

3.1. Chromatographic separation

The objectives of this study were to use HPLC-MS/MS to characterize the anthocyanin profile and quantify the anthocyanin content of sweetpotato leaves in response to various growth stages and conditions. Figure 4-2 shows the profile of anthocyanin peaks from sweetpotato leaves in HPLC chromatogram. No anthocyanin peaks were detectable in stems, P40, Murasaki, Bonita, and Beauregard flesh, but fourteen anthocyanins, including one newly discovered at retention times between 4 and 27 min, were found in the sweetpotato leaves. Of these, peaks 8 and 9 were the major anthocyanins and their peak areas appeared to cover more than half of total anthocyanin peak areas.

3.2. Mass spectrometric identification

Following HPLC separation, LC-MS/MS data were characterized by monitoring the molecular ion characteristics for each peak. Table 4-1 provides the *m*/*z* ratio of each intact anthocyanin and its fragment ions.

As shown in Table 4-1, Cyanidin (Dpd *m*/*z* 287) and Peonidin (Ptd *m*/*z* 301) were the two anthocyanidin aglycones detected in all four varieties of sweetpotato leaves. Islam et al. reported thirteen of the fourteen anthocyanins including Cyanidins (peaks 1, 3, 4, and 7-10) and Peonidin (peaks 2, 5, 6, and 11-13).[7] However, one new Peonidin (peak 14) was found in the all four varieties for the first time. The m/z ratio of a molecular ion with a fragment ion was captured within the scanning interval of 500–1200 m/z. The ions for peak 14 (Peonidin 3-caffeoyl-p-coumaryl sophoroside-5-glucoside; *m*/*z* 1095), produced three fragments at *m*/*z* 933, 463, and 301. Transition 1095-933 implied a loss of glucose (*m*/*z* 162), and 1095-463 a loss of 3-caffeoyl-p-coumaryl sophoroside (*m*/*z* 632). Transition 1095-301 produced Peonidin aglycone (*m*/*z* 301) caused by the loss of glucose and 3-caffeoyl-p-coumaryl sophoroside.

Fourteen anthocyanins were isolated and identified in all four varieties of sweetpotato leaves. Although this new anthocyanin could be detected in purple sweetpotato tubers (P40), [21] previous studies had not found Peonidin 3-caffeoyl-p-coumaryl sophoroside-5-glucoside in any sweetpotato leaves; this new anthocyanin was first reported in Murasaki sweetpotato leaves.

3.3. Anthocyanin content (AC) and total phenolic content (TPC) in sweetpotato leaves

AC at both slip and vine stages of all four varieties of sweetpotato leaves is given in Figure 4-3. No significant variation across the four varieties was found at the 1st slip stage. Results showed that Bonita produced the most AC during 1st and 2nd slip stages (280±30 mg /kg; $p<0.05$) while Beauregard had the richest AC at vine stage (592.5±86.4 mg/kg; $p<0.05$), reaching 592.5±86.4 mg/kg of the extracts, approximately 10 times more abundant than the amount of AC at 2nd slip stage. AC increased continuously in Bonita from 1st slip stage to vine stage, but P40 did not. Moreover, Bonita also showed high AC at vine stage, with 473.9±79.9 mg/kg,

significantly more than Murasaki and P40 leaves at the same stage ($p<0.05$). The changes in AC at P40 vine stage did not correspond to the changes observed in the other varieties. AC of P40 even decreased significantly from 2nd slip to vine stage.

The predominant anthocyanins were Cyanidin3-(6, 6'-dicaffeoyl-sophoroside)-5-glucoside, Cyanidin3-(6, 6'-caffeoyl-p-hydroxybenzoyl sophoroside)-5-glucoside, up to 60% of total anthocyanins within slip and vine stages. The new anthocyanin, Peonidin 3-caffeoyl-p-coumaryl sophoroside-5-glucoside accounted for approximately 1% of total anthocyanins. Xu et al. [22] reported the top three main anthocyanins in P40 flesh were Cyanidin3-(6,6'-caffeoyl-p-hydroxybenzoyl sophoroside)-5-glucoside, peonidin 3-caffeoyl sophoroside-5-glucoside, and Cyanidin3-(6,6'-caffeoylferuloyl-sophoroside) -5-glucoside, which accounted for half the total AC. Of these, Cyanidin 3-p-hydroxybenzoyl sophoroside-5-glucoside showed high levels in both flesh and leaves of P40.[22]

We found no sizeable changes in TPC in Murasaki from 1st slip stage to vine stage ($p<0.05$) (Figure 4-4); Beauregard at vine stage had the most abundant TPC (52.2±3 mg GAE/g DW), followed by Murasaki (44.6±2.4 mg GAE/g DW) and Bonita (40.8±3.3 mg GAE/g DW) ($p<0.05$) (Figure 4-4). Please note that TPC in P40 leaves did not change significantly for any stage, ranging from 36.3 ±1 mg GAE/g DW at 1st slip to 34.5 ± 1.4 mg GAE/g DW at vine stage.

In the field experiment, AC of Beauregard at slip stages did not differ significantly between high tunnel and open field environments ($p<0.05$). Similarly, TPC for Beauregard slip stages grown in the field was not higher than the high tunnel, indicating that Beauregard slips in high tunnels may not provide more health benefits than slips grown in the field ($p<0.05$). It has been reported that polyphenols like anthocyanins are sensitive to adverse environmental

conditions, including unfavorable temperature, light, pH, and humidity, so growing sweetpotatoes in a high tunnel does provide an easy and cost-effective way to establish more control over the growing environment and extend the growing season to enhance crop yield, quality, and profitability.[23] Despite the potential benefits of using the high tunnel at 1st and 2nd slip stages, additional study could determine the extent to which the vine stage might contribute to differing AC and TPC in the high tunnel study.

4. Conclusion

In conclusion, fourteen anthocyanins, including one new one, were identified and quantified by HPLC-MS/MS in the white-skin and white-fleshed Bonita, red-skin and orange-fleshed Beauregard, red-skin and white-fleshed Murasaki, and purple-skin and purple-fleshed P40. This study showed for the first time that anthocyanin levels are significantly affected by growth stage. Although the P40 tubers had the highest content of anthocyanins, Beauregard leaves at vine stage were richest in AC and TPC among the studied samples. AC increased continuously in Bonita from 1st slip stage to vine stage, but P40 did not have the same response. In the high tunnel studies, no significant differences in AC and TPC were found in Beauregard leaves grown in the high tunnels versus the open field. Hence, our overall results indicate that growth stages and/or environment affect anthocyanin content of various sweetpotato varieties, but that effect is highly variable and genotype-dependent.

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Table 4-1 Mass Spectrometric Data of Anthocyanins in Sweetpotato Leaves.

Peak Number	Retention (min)	Anthocyanins	[M+H] (m/z)	Fragment ions(m/z)
1	3.61	Cyanidin-3-sophoroside-5-glucoside	773	661,449,287
2	5.28	Peonidin-3-sophoroside-5-glucoside	787	595,433,271
3	6.23	p-hydroxybenzoylated (Cyanidin 3-sophoreside-5-glucoside)	893	731,449,287
4	6.96	Caffeoylated (Cyanidin 3-sophoroside-5-glucoside)	935	773,449,287
5	9.75	p-hydroxybenzoylated (Peonidin 3-sophoroside-5-glucoside)	907	745,463,301
6	10.29	Caffeoylated (Peonidin 3-sopheroside-5-glucoside)	949	787,463,301
7	11.31	Feruloylated (Cyanidin 3-sophoroside-5-glucoside)	949	787,449,287
8	17.90	Cyanidin 3-(6,6'-dicaffeoyl sophoroside)-5-glucoside	1097	935,449,287
9	17.57	Cyanidin 3-(6,6'-caffeoyl-p-hydroxybenzoyl sophoroside)-5-glucoside	1055	893,449,287
10	21.26	Cyanidin 3-(6,6'-caffeoyl-feruloyl-sophoroside)-5-glucoside	1111	949,449,287
11	22.23	Peonidin 3-(6,6'-dicaffeoyl-sophoroside)-5-glueoside	1111	949,463,301
12	22.85	Peonidin 3-(6,6'-caffeoyl-p-hydroxybenzoyl sophoreside)-5-glucoside	1069	907,463,301
13	25.30	Peonidin 3-(6,6'-caffeoyl-feruloyl sophoroside)-5-glucoside	1125	963,463,301
14	26.09	Peonidin 3-caffeoyl-p-coumaryl sophoroside-5-glucoside	1095	933,463,301

Figure Legends

Figure 4-1 Chemical structures of common anthocyanidins and anthocyanins.

Figure 4-2 Representative HPLC chromatograms of anthocyanins in sweetpotato leaves of vine stage: a)P40; b)Murasaki; c)Bonita; d) Beauregard, (the peak number corresponding to anthocyanin name is shown in table 1).

Figure 4-3 Anthocyanin content of Bonita, Beauregard, Beauregard high tunnel (Beauregard HT), Murasaki and P40 leaves under three different growth stages: 1st slip stage, 2nd slip stage and vine stage. (*Bars represent mean ± SE (n=4), Means in a cluster with the same letters are not significantly different at the 0.05 level.*)

Figure 4-4 Total phenolic content of Bonita, Beauregard, Beauregard high tunnel (Beauregard HT), Murasaki and P40 leaves under three different growth stages: 1st slip stage, 2nd slip stage and vine stage.(*Bars represent mean ± SE (n=4), Means in a cluster with the same letters are not significantly different at the 0.05 level.*)

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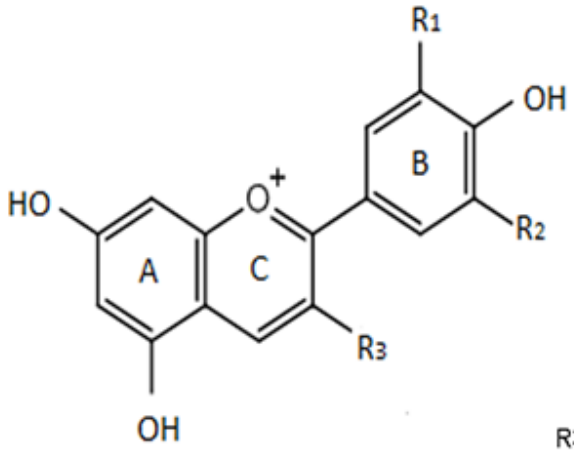

	R1	R2
Cyanidin	OH	H
Pelargonidin	H	H
Delphinidin	OH	OH
Petunidin	OCH3	OH
Peonidin	OCH3	H
Malvidin	OCH3	OCH3

R3 = Glucose, galactose, rhamnose, xylose, or arabinose

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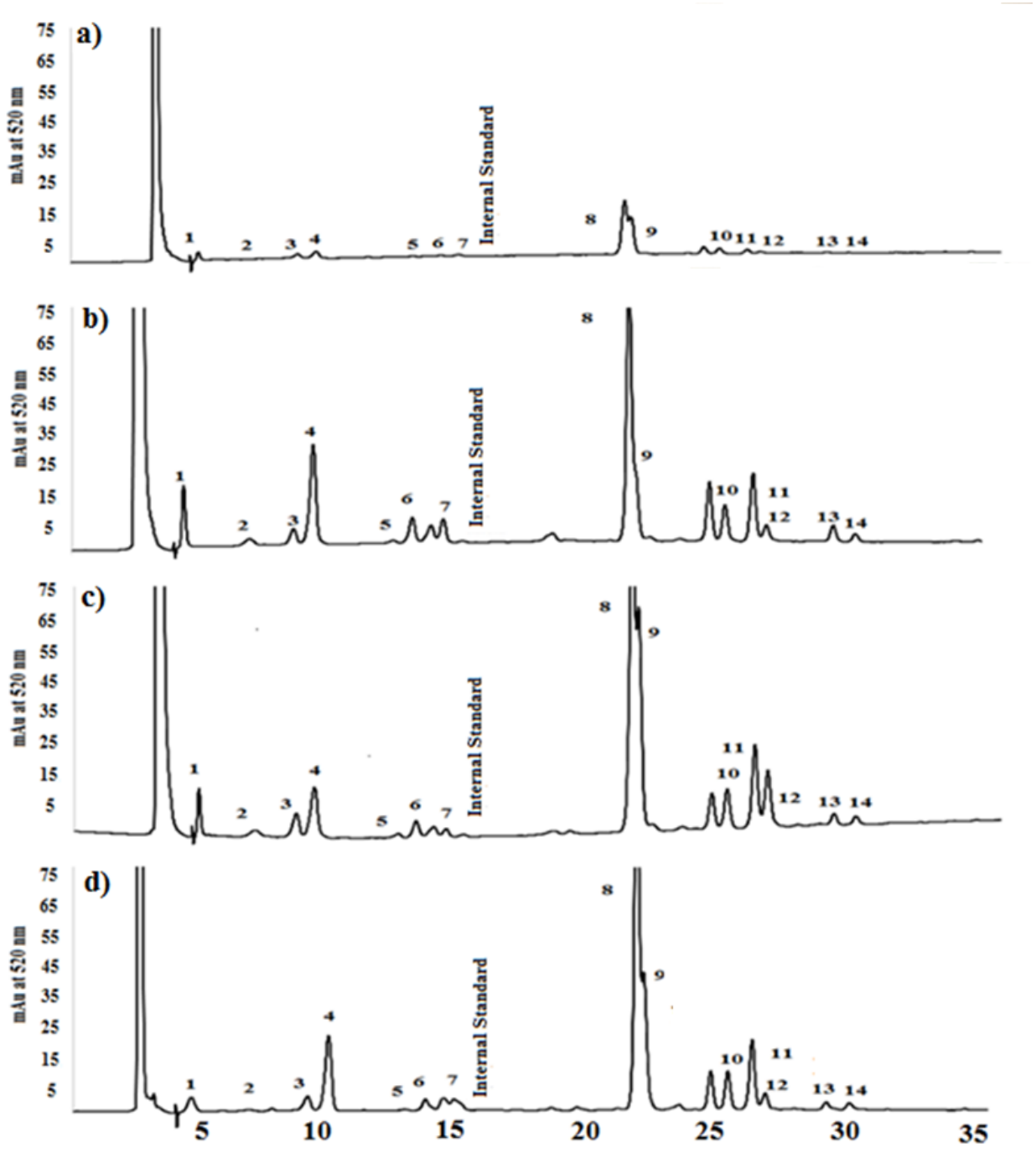

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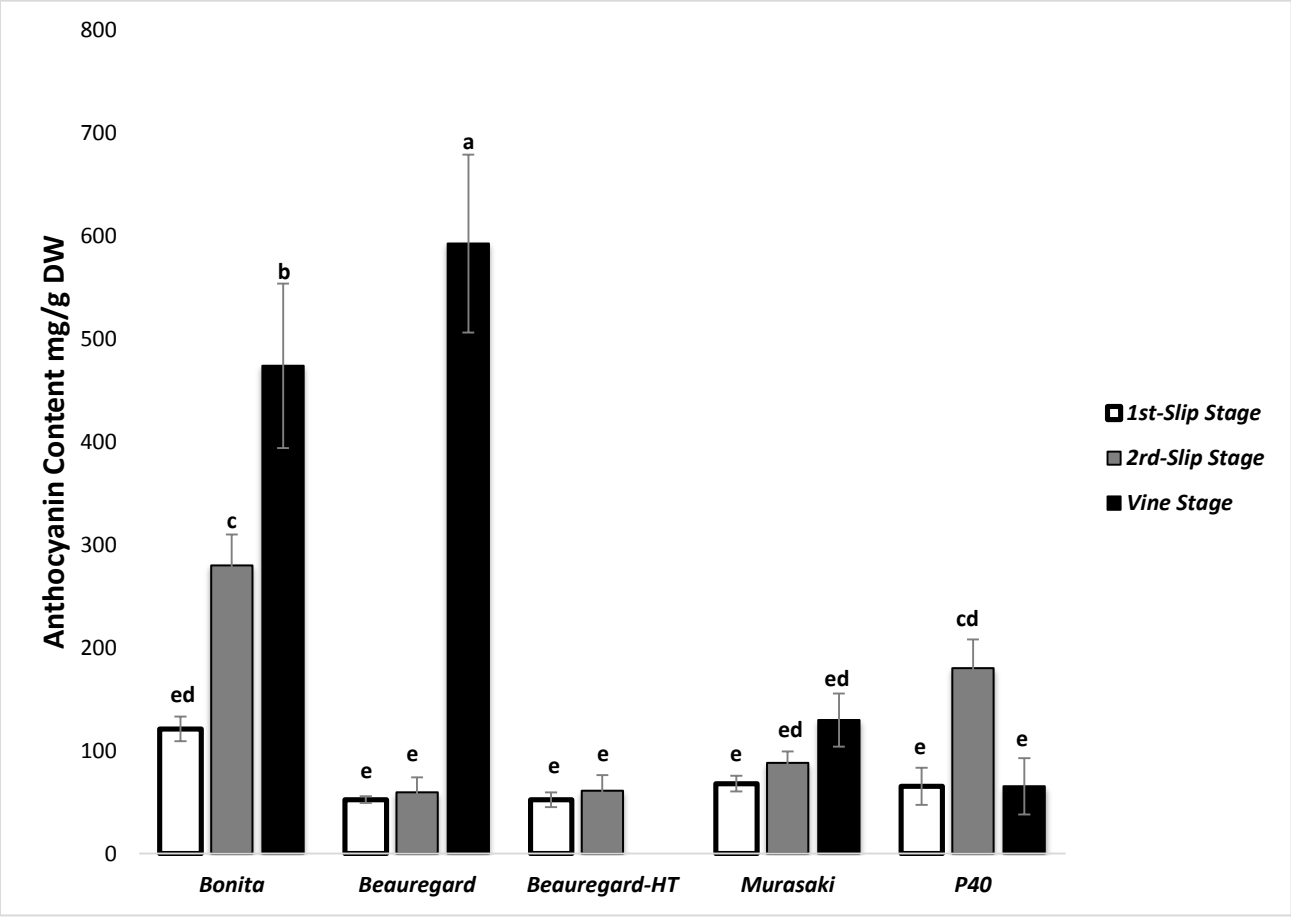

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