Influence of Plant Maturity on Anthocyanin Levels, Phenolic Composition, and Antioxidant Properties of 3 Purple Basil (*Ocimum basilicum* L.) Cultivars

Katherine R. McCance

A Thesis Submitted in Partial Fulfillment of the Requirements for Graduation with Honors in Biochemistry

Southwestern University, 2015

Approved: Emily D. Niemeyer

Dr. Emily D. Niemeyer, Honors Advisor
Department of Chemistry and Biochemistry

Kerry A. Bruns

Dr. Kerry A. Bruns, Committee Member
Department of Chemistry and Biochemistry

Benjamin A. Pierce

Dr. Benjamin A. Pierce, Committee Member
Department of Biology

M. Zewail-Foote

Dr. Maha Zewail-Foote, Department Chair
Department of Chemistry and Biochemistry

Influence of Plant Maturity on Anthocyanin Levels, Phenolic Composition, and Antioxidant Properties of 3 Purple Basil (*Ocimum basilicum* L.) Cultivars

Katie R. McCance

Department of Chemistry and Biochemistry, Southwestern University

April 2015

Abstract

In this study, we determined the effect of plant maturity on the total and individual anthocyanin and phenolic acid concentrations in three purple basil (*Ocimum basilicum* L.) cultivars as well as the contribution of these compounds to the herb's overall antioxidant capacity. Cultivar did not have a significant effect on phenolic acid concentrations, but plant maturity significantly influenced total phenolic levels ($p < 0.01$) as well as concentrations of individual phenolics, including caffeic ($p < 0.01$), *p*-coumaric ($p < 0.01$), caftaric ($p < 0.01$), gentisic ($p = 0.033$), chicoric ($p < 0.01$), and rosmarinic acids ($p < 0.01$). Cultivar ($p < 0.01$) and plant maturity ($p < 0.01$) significantly influenced the total anthocyanin content as well as the concentrations of the four most abundant individual anthocyanins (denoted as A-D). Plant maturity was also shown to have a significant effect on measured FRAP (ferric reducing antioxidant power) antioxidant capacities ($p < 0.01$). The individual phenolic and anthocyanin composition, in particular, was important in influencing the antioxidant properties. Overall, the cultivar and the maturity of basil at the time it is harvested play critical roles in the herb's phenolic and anthocyanin composition as well as its antioxidant properties.

Introduction

Plants and herbs have long been used not only as culinary ingredients but also as medicinal remedies in all parts of the world (Makri & Kintzios, 2007). Plants synthesize an enormous variety of organic compounds to fulfill their primary needs, such as photosynthesis, growth, and development, as well as to aid in secondary functions, such as attraction of

pollinators, UV protection, and defense (Crozier, Jaganath, & Clifford, 2006). Members of the Lamiaceae family of plants, such as basil (*Ocimum basilicum* L.), are abundant sources of a specific class of secondary metabolites known as phenolic acids (Zgórka & Glowniak, 2001). Basil is typically rich in rosmarinic, caffeic, caftaric, chicoric, and gentisic acids (Zgórka & Glowniak, 2001; Lee & Scagel, 2009; Kwee & Niemeyer, 2011; Flanigan & Niemeyer, 2014), possesses antioxidant properties, and confers an array of health benefits to humans with diets rich in plant-based foods (Hinneburg, Damien, & Hiltunen, 2006).

Purple varieties of basil contain anthocyanins, which belong to one of the largest classes of secondary metabolites known as flavonoids, and are responsible for red, blue, and purple pigmentation in plants (Phippen & Simon, 1998). Not only do anthocyanins serve a diverse range of functions for plants, from protecting against UV radiation to attracting pollinating insects (Gould, 2004), but they also provide numerous health-promoting effects to humans. These include anti-inflammatory, anticarcinogenic, and neuroprotective effects (He & Giusti, 2010; Kelsey, Hulick, Winter, Ross, & Linseman, 2011). Essential oils from purple basil were reported to protect cardiomyocytes against oxidative stress more effectively than the green basil cultivars Genovese and Lettuce Leaf, suggesting that variations in chemical composition and individual compounds among different cultivars influence protective effects (Danesi et al., 2008).

It is well established that many plants use similar pathways to synthesize secondary metabolites, but the quantities and types of phenolic acids and anthocyanins produced by a plant are greatly affected by the unique species and cultivar (Holton & Cornish, 1995). Cultivar's impact on the phenolic acid content and anthocyanin levels has been studied in basil (Kwee &

Niemeyer, 2011; Flanigan & Niemeyer, 2014) as well as in other dietary plants and foods, including purple corn and blueberries (Cuevas, Hillebrand, Antezana, & Winterhalter, 2011; Prior, Cao, Martin, Sofic, McEwen, O'Brien, Lischner, Ehlenfeldt, Kalt, Krewer, & Mainland, 1998). Phippen and Simon (1998) observed higher total anthocyanins in basil than in plums, red cabbage, and red raspberries.

Additionally, the influence of plant maturity at the time of harvest on phenolic acid concentrations, anthocyanin levels, and antioxidant properties has been previously studied in blueberries and black currants (Prior et al., 1998; Tabart, Kevers, Pincemail, Defraigne, & Dommes, 2006). Research by Phippen and Simon also included a growth study to investigate individual and total anthocyanin concentrations in purple basil over several months (Phippen & Simon, 1998). They found that anthocyanin composition varied in basil at different stages of its growth, and total anthocyanin levels increased prior to flowering then subsequently began to decrease. However, little is currently known specifically about the effect of plant maturity on individual anthocyanins and phenolic acids in purple basil and their contributions to basil's antioxidant capacity.

Basil synthesizes a range of secondary metabolites with potential disease-preventing properties and health benefits, and determining optimal levels of these qualities may enhance the herb's nutritional and medical value. The purpose of the current study is to determine how plant maturation influences the anthocyanin levels, phenolic composition, and antioxidant properties of purple basil cultivars. Specifically, the contributions of total and individual anthocyanin and phenolic acid concentrations to antioxidant capacities were compared among three purple basil varieties.

Materials and Methods

Chemicals and Reagents

The analytical standards kuromanin chloride (cyanidin 3-O-glucoside chloride), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), caffeic, caftaric, chicoric, gallic, gentisic, *p*-coumaric, and rosmarinic acids were of the highest purity and were obtained from Sigma-Aldrich (St. Louis, MO). Acetonitrile and methanol were HPLC grade and were purchased from Pharmco-AAPER (Brookfield, CT) and Sigma-Aldrich, respectively. Reagents such as iron (III) chloride hexahydrate, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), and formic acid were also purchased from Sigma-Aldrich. Sodium acetate trihydrate and hydrochloric acid were obtained from J.T. Baker (Phillipsburg, NJ) and Fisher Scientific (Fair Lawn, NJ), respectively. Sodium carbonate was purchased from EMD Chemicals, Inc. (Gibbstown, NJ), and Folin-Ciocalteu reagent was obtained from VWR International (West Chester, PA).

Plant Materials and Growth Conditions

Three *Ocimum basilicum* L. basil cultivars were selected for the study based on their known purple pigmentation and anthocyanin content. The cultivars were purchased from the following companies: 'Rosie' (RosB) from Richter's Herbs (Goodwood, Ontario), 'Purple Ruffles' (PR) from Johnny's Selected Seeds Company (Winslow, ME), and 'Sweet Petra Dark Red' (SPDR) from W. Atlee Burpee & Co. (Warminster, PA). Seeds were sown without fertilizer in 25 cm x 50 cm plastic seed trays containing Ferti-lome Seed and Cutting Mix (Cheek Garden Products, Austin, TX) on May 26, 2011. Plants were grown under natural light and temperature in a window sill and were watered once daily with tap water.

Four days after planting (May 30, 2011), seed trays were moved to the Southwestern University greenhouse, and germination occurred the next day (May 31, 2011). Nine days after germination (DAG), healthy seedlings were transferred to one gallon pots containing mesh bags filled with Ferti-lome Ready-to-Use Ultimate Potting Mix (Cheek Garden Products). Sixty six pots were organized in a randomized block design with 5 replicates per cultivar for each of five different harvest dates and were arranged so all pots received a similar amount of sunlight. Plants remained in the greenhouse (average temperature range: 25.2°C-46.5°C; average humidity range: 26.3%-88.4%) until the appropriate harvest date.

Plant Harvesting and Growth Analysis

Basil leaves from each cultivar were harvested on five unique dates: 9, 16, 23, 35, and 49 DAG. For the first harvest 9 days after germination, replicate seedlings were combined to obtain enough foliar mass for later analysis. For subsequent harvests, individual replicates contained leaves obtained from only a single plant. Leaves were immediately frozen in liquid nitrogen and stored at -80°C until processing.

When plants were harvested, the above-ground height, the total leaf mass, and total plant mass were measured for each plant. The average plant height, plant mass, and leaf mass were then determined for each harvest date. To quantify plant growth, the average growth per day since the previous harvest was calculated according to the following equation: $(x_x - x_{x-1}) / (DAG_x - DAG_{x-1})$. Average growth measurements (plant height, plant mass, or leaf mass) for a particular level of maturity are denoted as x_x, x_{x-1} represents the average measurement at the preceding

level of maturity, and DAG_x - DAG_{x-1} represents the days elapsed since the previous harvest date.

Basil Extract Preparation

To prepare leaves for analysis, a mortar and pestle were used to grind frozen leaves to a fine powder in liquid nitrogen, and 0.03-0.25 g of each sample was dried using vacuum centrifugation for 3.5 hours. Samples were immediately weighed to obtain their dry mass. Phenolic compounds were extracted according to a previous method (Nguyen & Niemeyer, 2008). Dried basil was incubated in 1.0 mL of 80% aqueous methanol and then shaken at room temperature for 15 hours. After centrifugation at 13,200 rpm for 30 minutes, the supernatant was removed, filtered using a 0.45 μm Whatman nylon filter, and stored at -80°C until further analysis. Anthocyanins were extracted by incubating dried basil in 0.5 mL of 0.2 M hydrochloric acid in methanol (acidified methanol) followed by shaking at room temperature for 40 minutes. After centrifugation at 13,200 rpm for 30 minutes, the supernatant was removed and stored at -80°C. This procedure was repeated for a total of 3 extractions, and the supernatants from each extraction were combined. All acidified methanolic extracts were filtered using a 0.45 μm Whatman nylon filter and stored at -80°C until analysis.

Total Phenolic and Anthocyanin Concentration Determination

Total phenolic concentration for each 80% methanolic sample was determined using a modified Folin-Ciocalteu (FC) colorimetric assay (Singleton & Rossi, 1965). A mixture of 150 μL of water, 100 μL of standard or 80% methanolic basil extract, 125 μL of FC reagent, and 625

μL of 0.2 M aqueous sodium carbonate was incubated in the dark at room temperature for 20 minutes (Flanigan & Niemeyer, 2014). Mixtures were then centrifuged at 13,200 rpm for 10 minutes. Using a BioRad Benchmark Plus microplate spectrophotometer (Hercules, CA), the absorbance of the supernatant was measured in triplicate at 735 nm on a 96-well plate against a blank consisting of 150 μL of water, 100 μL of 80% methanol, and 625 μL of 0.2 M aqueous sodium carbonate. Total phenolic concentration was quantified by comparing the average sample absorbance to a calibration curve of gallic acid in 80% methanol, ranging from 1.25 to 800.00 mg/L. Final concentrations were reported in gallic acid equivalents (GAE) in mg/g dry weight (DW).

Total anthocyanin concentrations in acidified methanolic extracts were determined using a modified version of a method developed by Abdel-Aal and Huel (1999). Prior to analysis, dilution with acidified methanol was performed on the extracts. The absorbance of the diluted extracts was measured in triplicate at 535 nm in a 96-well microplate against an acidified methanol blank. Total anthocyanin concentrations were quantified by comparison of the sample's average absorbance to a calibration curve of kuromanin chloride in acidified methanol, ranging from 0.98 to 62.50 mg/L. Final values were expressed in anthocyanin equivalents (AE) in mg/g DW.

Antioxidant Capacity Determination

Antioxidant capacities of acidified and 80% methanolic samples were determined using a modified ferric reducing antioxidant power (FRAP) assay (Benzie & Strain, 1996). The FRAP reagent was prepared daily and contained 20 mL of acetate buffer (0.3 M, pH 3.6), 2 mL of 20

mM aqueous iron (III) chloride, and 2 mL of 10 mM TPTZ dissolved in 40 mM HCl. For analysis, 25 µL of extract was incubated with 250 µL of FRAP reagent in a 96-well microplate and shaken at room temperature for 8 minutes. Samples were diluted with acidified or 80% methanol if needed prior to analysis. Absorbance of each extract (A_{sample}) was measured in triplicate at 593 nm and compared to a control ($A_{control}$), consisting of 25 µL of acidified or 80% methanol and 250 µL of FRAP reagent. The average difference in absorbance ($A = A_{sample} - A_{control}$) of each sample was compared to a calibration curve of trolox in acidified or 80% methanol, ranging from 20.00 to 1200.00 µM. Antioxidant capacities were expressed as trolox equivalent antioxidant capacities (TEAC) in mmol/100 g DW.

Individual Phenolic Acid and Anthocyanin Analysis

Analysis of individual phenolic acids (caffeic, caftaric, chicoric, gentisic, *p*-coumaric, and rosmarinic acids) was performed with a dual-pump Waters 717 Plus Autosampler HPLC system (Milford, MA) equipped with a 10 µL injection loop and a Phenomenex Kinetex (Torrance, CA) C-18 column (2.6 µm, 4.6 mm x 100 mm). Eluent A was 3% methanol and 1% formic acid in water, and eluent B was 0.1% formic acid in acetonitrile. Flow rate was held constant at 1.0 mL/min, and the following linear gradient conditions were used: hold at 95% A, 0-2 min; 95-75% A, 2-12 min; hold at 75% A, 12-17 min; 75-10% A, 17-18 min; hold at 10% A, 18-23 min; 10-95% A, 23-24 min; equilibrate at 95% A, 24-34 min. Dilutions with 80% methanol were necessary for some extracts before analysis. Detection occurred at 280 nm, and phenolic acids were identified in the samples by comparing their chromatographic retention times against analytical standards. Concentrations of the individual phenolic compounds were

quantified by integrating chromatographic peak areas and comparing them to external calibration curves for each phenolic acid (caffeic acid = 0.04-50.00 mg/L; caftaric acid = 0.26-50.00 mg/L; chicoric acid = 0.26-100.00 mg/L; gentisic acid = 0.87-65.00 mg/L; *p*-coumaric acid = 0.02-50.00 mg/L; and rosmarinic acid = 0.11-75.00 mg/L).

Analysis of individual anthocyanins was accomplished with a tri-pump Shimadzu HPLC system (Columbia, MD) equipped with a 50 µL injection loop and a Phenomenex Luna C-18 column (5 µm, 4.6 mm x 250 mm). The mobile phase consisted of 20% aqueous formic acid as eluent A, 100% acetonitrile as eluent B, and 100% water as eluent C. Flow rate was set at 0.3 mL/min, detection wavelength was 520 nm, and the concentration of eluent A was held constant at 50% of the mobile phase throughout the analysis. The following linear gradient conditions were used: 16% B, 0 min; 20% B, 10 min; 23% B, 35 min; 50% B, 50 min; 16% B, 52-62 min. A gentle stream of nitrogen gas was used to concentrate some samples prior to analysis.

Identification of individual anthocyanins was accomplished using liquid chromatography-mass spectrometry (LC-MS). An Agilent 1260 Infinity HPLC system, equipped with a Gemini C-18 column (3.5 µm, 2.1 mm x 50 mm) and 50 µL injection volume was interfaced with an Agilent 6530 Accurate-Mass QTOF mass spectrometer. The flow rate was set at 0.3 mL/min, and the following linear gradient program was used: 77.8% A, 10 min; 74.4% A, 35 min; 44.4% A, 50 min; 82.2% A, 62 min. Mass spectral analyses were completed in the positive electrospray ionization mode, and parameters such as gas temperature (350°C), gas flow (12 L/min), nebulizer pressure (50 psig), and fragmentor (180 units) were applied to optimize analysis. Mass spectra were collected from 100-3200 *m/z* with a scan rate of 1.00 spectra/sec.

Individual anthocyanins were identified based on their characteristic mass spectra and respective retention times. The four anthocyanins found in highest abundance within the basil cultivars (defined as anthocyanins A - D) were detected at 520 nm and quantified by integrating chromatographic peak areas and comparing them to an external calibration curve prepared from kuromanin chloride in acidified methanol, ranging from 0.31 to 25.00 mg/L. Final individual anthocyanin concentrations were reported as kuromanin equivalents (KE) in mg/g DW.

Statistical Analyses

All statistical analyses were performed using IBM SPSS Statistics, version 22.0 (SPSS Inc., Chicago, IL). All data are reported as the mean ± standard deviation and calculated using replicate samples. Prior to any statistical analyses, data sets were tested for normality using the Shapiro-Wilk test and homogeneity of variance using Levene's Test.

Two-way analysis of variance (ANOVA) was used to determine the individual and combined effect of cultivar and plant maturity on total and individual anthocyanin concentrations. Prior to analysis, a transformation (log for anthocyanins A and C; square root for anthocyanins B and D) was performed on data sets to achieve normality or homogeneity of variance. Statistical significance was determined at the $p < 0.05$ level using the appropriate post-hoc test (Bonferroni).

Friedman's two-way ANOVA, a non-parametric test, was used to determine the individual effects of cultivar and plant maturity on total phenolic concentrations, antioxidant capacities, and individual phenolic acid concentrations. Statistical significance was determined

using the post hoc Wilcoxon signed ranks test with the Bonferroni correction applied ($p < 0.0167$ for cultivar; $p < 0.01$ for plant maturity).

Hierarchical cluster analysis (HCA) was used to group plant maturities at the time of harvest based on similarities in their phenolic acid and anthocyanin compositions. One HCA analysis was performed using average concentrations of anthocyanins A-D, and a second HCA analysis was conducted on average concentrations of individual phenolic acids. Between-groups linkage was used with Euclidian distance measurement intervals, and z-scores were used to standardize the variables. Results of the analyses were then visualized using dendrogram plots.

Results and Discussion

Analysis of total and individual phenolic concentrations

Average total phenolic concentrations of the three cultivars harvested at varying levels of maturity are presented in Table 1, with concentrations ranging from 3.30 to 20.08 mg/g DW, both for Rosie basil harvested at 9 and 35 DAG, respectively. Overall, these concentrations are similar in range to values previously reported for eight purple basil cultivars by Flanigan & Niemeyer (2014). In their study, the average concentrations of Sweet Petra, Rosie, and Purple Ruffles cultivars were 22.01, 15.12, and 13.09 mg/g DW, respectively. Additionally, our values are similar to those reported for 15 green basil varieties, whose total phenolic concentrations ranged from 3.47 to 17.58 mg/g DW (Kwee & Niemeyer, 2011).

Cultivar did not have a significant effect on the total phenolic concentration. However, plant maturity at the time of harvest was determined to significantly influence the phenolic content. Basil harvested at 35 DAG had significantly higher total phenolic concentrations than

basil at any other level of maturity (9 DAG, $p = 0.008$; 16 DAG, $p = 0.001$; 23 DAG, $p = 0.001$; 49 DAG, $p = 0.003$), while basil at 49 DAG had significantly greater phenolic content than all other maturities except 35 DAG (9 DAG, $p = 0.008$; 16 DAG, $p = 0.003$; 23 DAG, $p = 0.003$).

It is interesting to highlight that basil harvested at 49 DAG had a significantly lower total phenolic level than basil at 35 DAG ($p = 0.003$). To investigate this relationship further, basil growth rates were compared between different plant maturities (Table 2). Basil experienced the greatest growth, in height and mass, between 23 and 35 DAG. For instance, plants grew at an average rate of 1.11 cm per day during that time period and correspondingly gained an average of 1.11 g of above-ground mass and 0.70 g of leaf mass each day. Although all basil continued to grow in terms of height and mass between 35 and 49 DAG, the rate of plant growth was not as high as that observed from 23 to 35 DAG. The growth-differentiation balance (GDB) hypothesis states that plants experience trade-offs between primary and secondary functions and metabolites, allowing resources and energy to be allocated toward the plant's most immediate needs (Herms & Mattson, 1992). During periods of intense growth, for instance, secondary metabolite production is predicted to slow in order to direct energy and substrate into growth and maturation processes for the plant. However, the growth trend in our study does not conform to the GDB's prediction since we observed high secondary metabolite production in the form of phenolic compounds during the period of most intense growth. Exceptions to the GDB exist, and in fact, secondary metabolite concentrations including low molecular weight phenolic compounds, are sometimes highest in early stages of growth or only synthesized at young stages of plant development (Herms & Mattson, 1992). Interestingly, a similar trend was observed for essential oil production in basil, with average essential oil content higher in young leaves than in

more mature leaves (Werker, Putievsky, Ravid, Dudai, & Katzir, 1993). Although we can not directly compare our results with those of Werker et al. (1993), essential oils contain secondary metabolites and therefore factors governing essential oil production throughout basil's maturation may be similar to those influencing phenolic synthesis.

The six most abundant phenolic acids (caffeic, *p*-coumaric, caftaric, gentisic, chicoric, and rosmarinic acids) detected within the basil cultivars were quantified using high-performance liquid chromatography and the average concentrations are displayed in Table 3. Plant maturity at the time of harvest was determined to significantly affect the concentrations of all six phenolic acids analyzed (rosmarinic, chicoric, caftaric, *p*-coumaric, caffeic acids, $p < 0.01$; gentisic acid, $p = 0.033$), however, cultivar's effect was significant only for *p*-coumaric acid ($p = 0.010$).

Caffeic and *p*-coumaric acids are cinnamic acid derivatives which are commonly found in herbs, including the Lamiaceae family (Zgórka & Glowniak, 2001). Caffeic acid is a phenolic acid widely distributed throughout the plant kingdom, however, it was the least abundant phenolic acid included in our study, with average concentrations ranging from 0.036 to 0.22 mg/g DW at 9 and 35 DAG, respectively. The values we observed were similar to those reported in the literature for other basil varieties (Flanigan & Niemeyer, 2014; Kwee & Niemeyer, 2011), with the exception of the Gecofure cultivar (Kwee & Niemeyer, 2014), which had an average caffeic acid concentration of 0.77 mg/g DW. A previous study (Javanmardi, Khalighi, Kashi, Bais, & Vivanco, 2002) also found caffeic acid to be present in low concentrations in Iranian basil, with levels no greater than approximately 0.003 mg/g DW. The common plant phenolic, *p*-coumaric acid, has generally been detected in low concentrations in basil. A study of Iranian basil identified *p*-coumaric acid in quantities less than 0.35 mg/g DW in the leaves and flowers

of several basil accessions (Javanmardi et al., 2002). Our concentrations were within the same range, with average values of 0.019 mg/g DW (9 DAG) to 0.45 mg/g DW (35 DAG). Cultivar was shown to have a significant effect on *p*-coumaric acid concentrations ($p = 0.01$), with Rosie having significantly lower levels (0.11 mg/g DW) than Purple Ruffles (0.20 mg/g DW; $p = 0.012$) and Sweet Petra Dark Red (0.16 mg/g DW; $p = 0.001$).

Caftaric acid was first identified in basil by Lee and Scagel (2009), who measured concentrations of 0.17 mg/g of fresh weight (FW) for Sweet Basil, 0.02 mg/g FW for Thai Basil, and only 0.004 mg/g FW for Purple Petra. It is difficult, though, for us to make comparisons with Lee and Scagel's findings because they analyzed fresh plant tissue (we analyzed dried) and water content can result in lower reported phenolic concentrations. In our study, average caftaric acid levels ranged from 0.086 mg/g DW (9 DAG) to 0.85 mg/g DW (35 DAG), and overall the values we observed are slightly higher than those previously reported in purple cultivars, where the highest level of caftaric acid was 0.12 mg/g DW for the cultivar Amethyst Improved (Flanigan & Niemeyer, 2014). A study of green basil varieties, however, showed a similar range of caftaric levels (Kwee & Niemeyer, 2011).

Plant maturity had a significant effect on caffeic, *p*-coumaric, and caftaric acid concentrations. Caffeic and *p*-coumaric acid concentrations were significantly lower at the earliest level of maturity than all other maturities ($p = 0.008$). Basil at 35 and 49 DAG had higher concentrations of caffeic, *p*-coumaric, and caftaric acids than basil at 9, 16, and 23 DAG (35 DAG: 9 DAG, $p = 0.008$; 16 DAG, $p = 0.001$; and 23 DAG, $p = 0.001$; 49 DAG: 9 DAG, $p = 0.008$; 16 DAG, $p = 0.003$; and 23 DAG, $p = 0.003$). Additionally, basil at 49 DAG had significantly lower concentrations of *p*-coumaric acid than basil at 35 DAG ($p = 0.003$).

Gentisic acid is commonly identified in green plants (Griffiths, 1959), and previous studies in the literature have reported gentisic acid in basil (Javanmardi et al., 2002; Proestos, Chorianopoulos, Nychas, & Komaitis, 2005; Flanigan & Niemeyer, 2014). Our average gentisic acid concentrations ranged from 0.80 (9 DAG) to 2.11 mg/g DW (35 DAG), which are comparable to levels observed by Flanigan and Niemeyer (2014), who reported gentisic acid as the dominant phenolic acid in two purple basil varieties. Our gentisic acid concentrations, though, are close to 100 times greater than those reported in Iranian accessions of basil (less than 0.02 mg/g DW) (Javanmardi et al., 2002). In our study, concentrations at 35 DAG were significantly higher than those at 16 DAG ($p = 0.003$), however, no other significant differences between maturity levels existed for this phenolic acid.

Chicoric acid, a phenolic acid only recently identified in basil, was reported as the second most abundant phenolic by Lee and Scagel (2009), who observed an average of 0.89 mg/g FW in Thai Basil. Chicoric acid was also the second most abundant phenolic acid identified in our study, and average values ranged from 0.13 mg/g DW (9 DAG) to 3.55 mg/g DW (35 DAG). Basil harvested at 9 DAG had significantly lower chicoric acid concentrations than any other level of maturity ($p = 0.008$), while concentrations at 35 DAG were greater than all other maturity levels (9 DAG, $p = 0.008$; 16 DAG, $p = 0.001$; 23 DAG, $p = 0.001$; 49 DAG, $p = 0.003$). Basil at 49 DAG also contained higher chicoric concentrations than basil at any other level of maturity (9 DAG, $p = 0.008$; 16 DAG, $p = 0.003$; 23 DAG, $p = 0.003$), except basil at 35 DAG. Our chicoric acid values are greater than those reported in the literature by Lee and Scagel (2009), however, this result is expected because dried leaves express higher phenolic concentrations than fresh plant mass.

Herbs are the only dietary source of rosmarinic acid - a caffeic acid ester - and its presence in herbs is associated with a multitude of health benefits (Crozier, Jaganath, & Clifford, 2006; Petersen & Simmonds, 2003). It is typically one of the most abundant phenolic acids found in basil, and in our study, it was the phenolic present in the largest quantities with average concentrations ranging from 1.31 (9 DAG) to 21.31 mg/g DW (35 DAG). Plant maturity was shown to have a significant effect on rosmarinic acid levels, with basil at 9 DAG having lower concentrations than all other levels of maturity ($p = 0.008$), and 35 DAG having greater levels than any other plant maturity (9 DAG, $p = 0.008$; 16 DAG, $p = 0.001$; 23 DAG, $p = 0.001$; 49 DAG, $p = 0.003$). Our range of values is much larger than rosmarinic acid concentrations reported for purple basil within the literature, from 0.66 to 1.60 mg/g DW (Flanigan & Niemeyer, 2014). However, rosmarinic acid levels found in Iranian accessions demonstrated concentrations much greater than our own, with some accessions having close to 100 mg/g DW (Javanmardi et al., 2002). Variations in rosmarinic acid levels, however, are highly dependent on the environmental conditions that plants are exposed to during growth, which may explain the large differences in the reported values for this phenolic acid (Javanmardi et al., 2002).

Total and individual anthocyanin concentrations

The average total anthocyanin concentrations of basil cultivars harvested at varying maturities are presented in Figure 1. Concentrations ranged from 2.07 mg/g DW for Sweet Petra Dark Red (9 DAG) to 9.72 mg/g DW for Purple Ruffles (35 DAG). These values are lower than those reported by Flanigan and Niemeyer, who observed total anthocyanin levels ranging from 7.55 to 16.6 mg/g DW for the cultivars Rubin and Purple Ruffles (Richter's Herbs), respectively

(Flanigan & Niemeyer, 2014). Although all basil from their study was harvested at 39 DAG, we did not observe concentrations as high as Flanigan and Niemeyer's even in our basil harvested at 35 and 49 DAG. However, our average total anthocyanin concentrations were higher than those observed by Phippen and Simon, who reported Purple Ruffles from Johnny's Selected Seeds with an average concentration of 0.16 mg/g FW (Phippen & Simon, 1998). The substantially lower concentration, though, may be due to variation in plant material and extraction methods. Some studies, including Phippen and Simon's, used fresh plant tissue, whereas in our study, and others as well, anthocyanins were extracted from dried leaves. Additionally, extracting from stems and flowers, as Phippen and Simon did, may underestimate total anthocyanin concentrations, as anthocyanin accumulation in the leaves of basil is the largest contributor to purple basil's color (Phippen & Simon, 1998; Chalker-Scott, 1999).

Cultivar was determined to have a significant effect on the total anthocyanin concentrations ($p < 0.01$). Purple Ruffles had significantly higher anthocyanin levels than both Rosie ($p = 0.003$) and Sweet Petra Dark Red ($p < 0.01$). Rosie and Sweet Petra Dark Red, however, were not statistically different from one another. These results agree with Phippen and Simon's observation that large-leaf varieties of purple basil, such as Purple Ruffles, contain greater anthocyanin concentrations than other varieties (Phippen & Simon, 1998). While purple cultivars of basil have been shown to contain significantly higher concentrations of anthocyanins than green varieties, large differences in anthocyanin levels may also exist between purple cultivars (Kwee & Niemeyer, 2011). Kwee and Niemeyer found that the average total anthocyanin concentration of Petra Dark Red was almost 10 times greater than that of Ararat and Osmin, which are both purple cultivars. Additionally, Flanigan and Niemeyer determined Purple

Ruffles to have significantly higher anthocyanin content than one other purple cultivar, Rubin (Flanigan & Niemeyer, 2014).

Harvest date also significantly influenced total anthocyanin concentrations ($p < 0.01$). Basil at 9 DAG had significantly lower total anthocyanin concentrations than basil at 16 ($p < 0.01$), 23 ($p = 0.001$), and 35 DAG ($p < 0.01$), while basil at 35 DAG had higher levels than basil at 9 ($p < 0.01$), 23 ($p = 0.029$), and 49 DAG ($p < 0.01$). Similarly, total anthocyanin concentrations in blueberries have been shown to vary with maturity (Prior et al., 1998). The total anthocyanin levels of mature berries were more than 3.5 times greater than those of berries that were less mature by 49 days. In our study, the largest increase in average anthocyanin levels was observed for Purple Ruffles between 9 and 35 DAG (3.66 mg/g DW to 9.72 mg/g DW). The total anthocyanin content of basil at 35 DAG was more than 2.5 times greater than basil at 9 DAG. Although this increase was not as great as the one observed in blueberries, variations in harvest maturities (49 day difference with blueberries, 26 day difference for our basil) as well as the unique growth properties of blueberries compared to basil most likely account for this result. Additionally, our values are much higher than those reported by Phippen and Simon in their growth study of Burpee's Purple Ruffles (Phippen & Simon, 1998). This cultivar had average total anthocyanin concentrations of 0.12 mg/g FW and 0.16 mg/g FW after being harvested at 8 and 30 DAG, while we observed a much greater range between basil harvested at 9 and 35 DAG. However, as mentioned previously, extraction methods and selection of plant material may contribute to the lower anthocyanin values observed in Phippen and Simon's study. It is interesting to note that basil harvested at 49 DAG had significantly lower total anthocyanin levels than basil harvested at 35 DAG in our study, because in Phippen and Simon's study

(1998), anthocyanin concentrations steadily increased in Purple Ruffles until just prior to flowering (90 DAG), and flowering does not initiate until 14 weeks for most cultivars of *Ocimum basilicum* L. (Darrah, 1974). However, as mentioned before, certain developmental demands of the basil plants between 35 and 49 DAG may explain the decrease in total anthocyanin concentrations. It was also determined that the interaction between cultivar and plant maturity at the time of harvest had a significant effect on total anthocyanin levels (p = 0.048). This result indicates that plant maturity and cultivar have a unique combined effect on total anthocyanin levels.

The concentrations of the four most abundant individual anthocyanins (referred to as anthocyanins A-D) were determined using high performance liquid chromatography and their general structures were identified using mass spectrometry. The presence of the *m/z* 287 peak that is characteristic of the cyanidin aglycon was not detected in anthocyanins A-D. By comparing mass spectrometric peaks, chromatographic elution order, and anthocyanin molecular ion, $[M+H]^+$, peaks reported in the literature, it was determined that anthocyanins A-D in our study were similar to those found by Phippen and Simon (1998) as well as Flanigan and Niemeyer (2014). Anthocyanins A (retention time, t_r = 29 min; $[M+H]^+$ = *m/z* 1081) and C (t_r = 38 min; $[M+H]^+$ = *m/z* 1065) were likely cyanidin-based *p*-coumaryl acids, and anthocyanins B (t_r = 34 min; $[M+H]^+$ = *m/z* 1167) and D (t_r = 46 min; $[M+H]^+$ = *m/z* 1151) were assigned as cyanidin-based *p*-coumaryl and malonyl acids.

Average concentrations of individual anthocyanins, measured in kuromanin equivalents (KE), are displayed in Table 4. Anthocyanins A-D were detected in all cultivars at all levels of maturity that were studied. Anthocyanin C was the most abundant, with concentrations ranging

from 0.41 mg/g DW for Sweet Petra Dark Red (9 DAG) to 2.02 mg/g DW for Purple Ruffles (23 DAG). In contrast, anthocyanin B was present in the lowest quantities, ranging from 0.05 mg/g DW for Sweet Petra Dark Red (9 DAG) to 0.44 mg/g DW for Purple Ruffles (35 DAG). Although Phippen and Simon did not quantify the concentrations of the individual anthocyanins detected in their basil cultivars, the relative chromatographic peak heights they reported correlate with the abundance of anthocyanins detected in our study (Phippen & Simon, 1998). For instance, the compound that we identified as anthocyanin C was present in the highest quantity in their study, and the chromatographic peak that we denote as anthocyanin B was present in lowest quantity in Phippen and Simon's study. However, the concentration of anthocyanin A that we observed for each cultivar at 35 DAG was about twice as high as those reported by Flanigan and Niemeyer (2014). Additionally, our values were slightly lower overall for anthocyanin B, slightly greater for anthocyanin C, and similar to anthocyanin D when compared to Flanigan and Niemeyer's (2014) results.

Cultivar had a significant effect on the concentrations of anthocyanins A-D, with Purple Ruffles producing higher levels of individual anthocyanins than Sweet Petra Dark Red for all anthocyanins (A, $p = 0.001$; B, $p < 0.01$; C, $p < 0.01$; D, $p < 0.01$), and higher levels than Rosie for anthocyanins C ($p < 0.01$) and D ($p = 0.024$) only. Plant maturity at the time of harvest was also determined to significantly affect the concentrations of anthocyanins A-D, with basil harvested at 35 DAG containing greater individual anthocyanin concentrations than basil that had been harvested at 9 DAG (A, $p < 0.01$; B, $p < 0.01$; C, $p = 0.003$; D, $p = 0.007$). Additionally, basil at 9 and 49 DAG had lower concentrations than basil at 16 (9 DAG, $p = 0.001$; 49 DAG, $p = 0.027$) and 23 DAG (9 DAG, $p = 0.001$; 49 DAG, $p = 0.023$) for

anthocyanin C, and lower than 16, 23, and 35 DAG in anthocyanins B (16 DAG, $p = 0.001$; 23 DAG, $p = 0.001$; 35 DAG, $p < 0.01$) and D (9 DAG: 16 DAG, $p < 0.01$; 23 DAG, $p < 0.01$; and 35 DAG, $p = 0.007$; 49 DAG: 16, 23, and 35 DAG, $p < 0.01$). The interaction of cultivar and harvest date was also found to be significant in influencing the concentrations of anthocyanins B ($p = 0.005$) and D ($p = 0.021$) only. As mentioned previously, an interaction indicates that the combined effect of plant maturity at the time of harvest and cultivar influences the concentrations of these individual anthocyanins.

Hierarchical Cluster Analysis

Hierarchical cluster analysis was used to group plant maturities at harvest based on similarities in their phenolic acid and anthocyanin profiles, and the results are displayed in Figure 2. When plant maturities were clustered based on similarities in their individual phenolic acid profile (Figure 2A), three groups resulted. The largest cluster contained basil harvested at 9, 16, and 23 DAG and was characterized by low levels of all six phenolic acids (caftaric, gentisic, caffeic, *p*-coumaric, chicoric, and rosmarinic acids). A second cluster contained only basil harvested at 35 DAG, which had high levels of all six phenolic acids. The third cluster contained basil harvested at 49 DAG and had high concentrations of caftaric and caffeic acids, moderate levels of chicoric acid, and low concentrations of gentisic, *p*-coumaric, and rosmarinic acids.

In grouping the maturities based on similarities in anthocyanin A-D composition (Figure 2B), the largest cluster contained basil harvested at 16 and 23 DAG, which was characterized by moderate levels of anthocyanins A and B and high levels of anthocyanins C and D. Basil harvested at 9 DAG was contained in its own group and had low concentrations of anthocyanins

A-D. A third cluster contained basil harvested at 35 DAG, which had high levels of anthocyanins A-D, and the fourth cluster contained basil harvested at 49 DAG, which contained moderate levels of anthocyanins A and C but low concentrations of anthocyanins B and D.

Comparing our hierarchical cluster analysis results for individual anthocyanins and individual phenolic acids suggests that different relationships exist in the production of these secondary metabolites. Basil harvested at 9 DAG belonged to clusters characterized by low concentrations of all four anthocyanins and all six phenolic acids, while basil harvested at 35 DAG belonged to clusters containing high levels of all anthocyanins and all phenolic acids. Basil at 49 DAG, however, was placed in a cluster that had low to moderate concentrations of anthocyanins A-D, but contained moderately high levels of two phenolics, caftaric and caffeic acids. This observed trade-off may suggest that anthocyanin synthesis decreases when phenolic acid production is high. Conversely, basil harvested at 16 and 23 DAG contained moderate to high levels of anthocyanins A-D, but low levels of all six phenolic acids. This, too, implies that at certain stages of growth, basil may experience a trade-off in metabolite production, which was similarly observed by Flanigan and Niemeyer (2014) when they compared individual anthocyanin and phenolic acid profiles of purple basil. It is possible that if fewer resources are entering anthocyanin synthetic pathways, then more substrate may be available to produce other secondary metabolites, such as phenolic acids, and vice versa (Stamp, 2003).

Antioxidant capacities

Extractability of phenolic acids and anthocyanins is greatly influenced by factors such as temperature, pH, and the composition of extraction solvents. Organic solvents such as methanol

and ethanol are widely used, and it is advantageous to perform anthocyanin extraction using acidified solvents (Gachovska, Cassada, Subbiah, Hanna, Thippareddi, & Snow, 2010; Wrolstad, Durst, Giusti, & Rodriguez-Saona, 2001). This is because the flavylium cation form of anthocyanins is more stable under acidic conditions than the hemiketal and quinoidal forms which are favored at higher pHs (Wrolstad, Durst, Giusti, & Rodriguez-Saona, 2001). Although extraction methods are optimized to maximize the extraction yield for a desired analyte, extraction methods, including those conducted in our study, are not entirely selective processes. Phenolic compounds and anthocyanins, for example, are both extractable in acidified methanol. However, extraction using 80% methanol has a higher selectivity for phenolic acids, because anthocyanins are unstable and susceptible to degradation under non-acidic conditions. In this study, we measured the FRAP antioxidant capacity of both 80% methanolic and acidified methanolic extracts. The FRAP antioxidant capacity of the 80% methanolic extracts will therefore primarily measure the antioxidant activity of phenolic compounds, while FRAP antioxidant capacities of the acidified methanolic extracts are likely to measure activities of anthocyanins as well as other phenolic acids that were co-extracted (Flanigan & Niemeyer, 2014).

Table 5 presents the average antioxidant capacities of the acidified methanolic and 80% methanolic basil extracts as measured by the FRAP assay. The FRAP assay was selected because it is conducted under acidic conditions, which stabilizes anthocyanins, and because it is simple and commonly used in plant extract analysis (Huang, Ou, & Prior, 2005). Average antioxidant capacities for acidified methanolic extracts ranged from 3.50 to 28.73 mmol/100 g DW for Sweet Petra Dark Red (9 DAG) and Rosie (35 DAG), respectively. Although cultivar did not

significantly affect the antioxidant capacities, the maturity of basil at harvest did have a significant influence ($p < 0.01$). Basil at 9 DAG had significantly lower antioxidant capacities than basil at any other maturity (16, 23, 35, and 49 DAG, $p = 0.008$), and basil at 35 and 49 DAG had significantly higher antioxidant capacities than basil harvested at 16 (35 DAG, $p = 0.001$; 49 DAG, $p = 0.003$) and 23 DAG (35 DAG, $p = 0.001$; 49 DAG, $p = 0.004$). However, antioxidant capacities did not differ between basil harvested at 35 and 49 DAG. The antioxidant values of basil at 35 DAG are comparative to those reported by Flanigan and Niemeyer (2014), whose antioxidant capacities for acidified methanolic basil extracts ranged from 24.6 (Johnny's Purple Ruffles) to 38.4 mmol/100 g DW (Purple Delight).

Extraction of phenolic compounds was performed using 80% methanol, and the average antioxidant capacity for each extract was determined. Antioxidant capacities ranged from 2.18 to 35.50 mmol/100 g DW for Purple Ruffles (9 DAG) and Rosie (35 DAG), respectively. Cultivar and maturity at harvest were both significant in influencing the measured FRAP antioxidant capacities. Purple Ruffles had a higher average antioxidant capacity than Rosie ($p = 0.002$) and Sweet Petra Dark Red ($p < 0.01$). Additionally, FRAP antioxidant capacity values at 35 DAG were greater than those at any other level of maturity (9 DAG, $p = 0.008$; 16 DAG, $p = 0.001$; 23 DAG, $p = 0.001$; 49 DAG, $p = 0.008$). Basil at 49 DAG had greater levels than 9 ($p = 0.008$), 16 ($p = 0.003$), and 23 DAG ($p = 0.003$), and basil at 9 DAG also had significantly lower antioxidant activity than basil at 16 DAG ($p = 0.008$).

The FRAP antioxidant capacities of the acidified methanolic and 80% methanolic samples in this study are greater than antioxidant capacities reported in the literature for green cultivars. For example, Kwee and Niemeyer (2011) found FRAP antioxidant capacities of 80%

methanolic extracts ranging from 0.28 mmol/100 g DW for Sweet Dani Lemon to 11.46 mmol/ 100 g DW for Gecofure. However, the greater values that we observed may be due to higher total anthocyanin concentrations in our purple varieties, which may contribute differently to antioxidant activity than phenolic compounds alone.

Plotting the average total anthocyanin concentration against the FRAP antioxidant capacity for each acidified methanolic extract (data not shown), showed a very low level of correlation ($y = 1.88x + 2.61$ by linear least squares analysis; $R^2 = 0.1876$). This poor correlation is evident in several instances. For example, although Purple Ruffles at 35 DAG was shown to contain the highest total anthocyanin concentration of all cultivars and levels of maturity, it possessed the fifth highest FRAP antioxidant capacity for acidified extracts. Rosie at 49 DAG had the second lowest total anthocyanin concentration, yet contained the second highest FRAP antioxidant capacity. Similarly, Flanigan and Niemeyer (2014) observed low correlation between total anthocyanin concentrations and their associated FRAP antioxidant capacities. These results suggest that measured antioxidant capacities are not strongly determined by total anthocyanin content.

A similar plot of total phenolic concentration as a function of FRAP antioxidant capacity for each 80% methanolic extract (data not shown) revealed a much greater correlation ($y = 1.72x - 3.07$ by linear least squares analysis; $R^2 = 0.8088$). High correlation between total phenolic concentration, which was measured by the Folin-Ciocalteu assay, and the FRAP antioxidant capacity of 80% methanolic extracts is expected, though, because both assays use an electron transfer-based mechanism (Huang, Ou, & Prior, 2005). Total anthocyanin concentrations,

however, were measured using a spectrophotometric method and do not function by a chemistry that is similar to the FRAP assay.

Because phenolic acids may be co-extracted with anthocyanins in acidified methanolic extracts, the combined influence of anthocyanin and phenolic concentrations on antioxidant capacities in acidified methanol extracts was investigated. The sum of the total anthocyanin and phenolic concentrations was plotted against the FRAP antioxidant capacity for each of the acidified methanolic extract (data not shown), and a moderately high degree of correlation was observed ($y = 1.16x - 3.28$ by linear least squares analysis; $R^2 = 0.7921$). This correlation was much higher than the correlation with total anthocyanin concentrations alone, and it was similar to the correlation between the antioxidant capacity of 80% methanolic extracts and total phenolic concentrations alone. This suggests the importance of phenolics in influencing the antioxidant capacity of basil relative to anthocyanins.

Examination of hierarchical cluster analysis results in the context of antioxidant capacities of acidified methanolic extracts reveals several interesting relationships. Basil at 9 and 35 DAG were placed in clusters characterized by low and high levels of all individual anthocyanins and phenolic acids, respectively, and they also correspondingly had the lowest and highest average antioxidant capacities. Basil at 16 and 23 DAG was characterized by containing moderate to high levels of anthocyanins A-D, and low levels of all phenolic acids. They also had relatively low measured average antioxidant capacities. However, basil at 49 DAG possessed low to moderate concentrations of anthocyanins A-D, had high levels of two phenolics, and expressed some of the highest average antioxidant capacities reported in our study. Although total anthocyanin concentrations were poorly correlated with their associated antioxidant

capacities, individual anthocyanin and phenolic composition seems to have a greater influence on measured average antioxidant capacities of acidified methanolic extracts. Additionally, this result implies that phenolic acids present in the acidified methanolic extracts may be the largest contributors to measured antioxidant capacities in acidified extracts. Overall, these results suggest that total as well as individual phenolic concentrations serve as appropriate indicators of the antioxidant properties of basil, whereas antioxidant capacities seem to be less directly dependent on anthocyanin concentrations.

Conclusions

In this study, we have shown that cultivar and plant maturity have significant effects on the anthocyanin and phenolic composition as well as the antioxidant capacity of purple basil. Plant maturity significantly influenced the total and individual anthocyanin and phenolic concentrations as well as the measured antioxidant capacities, while cultivar had a significant effect on total and individual anthocyanin concentrations and antioxidant capacities. However, cultivar did not have a significant effect on total or individual phenolic acid concentrations, except for *p*-coumaric acid.

Hierarchical cluster analysis grouped plant maturities into four categories, and further analysis of individual anthocyanins and phenolic acids with relation to their acidified methanolic antioxidant capacities revealed that phenolic acids were important in influencing antioxidant capacities. The first group (9 DAG), characterized by low anthocyanin and phenolic content, had correspondingly low average antioxidant capacity; the second group (16 and 23 DAG), with moderate to high anthocyanin concentrations and low levels of all phenolic acids, also had low

average antioxidant capacities; the third group (35 DAG), containing high concentrations of all anthocyanins and phenolic acids, had high antioxidant capacities; and the fourth group (49 DAG), with low to moderate levels of anthocyanins but high levels of caftaric and caffeic acids, had relatively high antioxidant capacities. These results not only suggest that a trade-off between individual anthocyanin and phenolic acid production exists in basil at certain stages of its growth, but also that the individual phenolic composition has a large effect on the measured antioxidant capacities. Overall, the cultivar and the maturity of basil at the time it is harvested play critical roles in the herb's phenolic and anthocyanin composition as well as its antioxidant properties.

Acknowledgements

Financial support for this work was generously provided by the Howard Hughes Medical Institute, the Robert A. Welch Foundation, and Southwestern University. The authors would like to thank Mr. Patrick Flanigan for growing and harvesting the basil for this research. We are also grateful for Dr. Brandon Canfield (Northern Michigan University) and Mr. Maxime Boneza for their help in HPLC analysis. We also wish to thank Ian Riddington and Jordan Disner of the Mass Spectrometry Facility (Department of Chemistry and Biochemistry, University of Texas at Austin) for their contribution in confirming anthocyanin identities with mass spectrometry.

References

Abdel-Aal, E.S.M.; Huel, P. A rapid method for quantifying total anthocyanins in blue aleurone and purple pericarp wheats. *Cereal Chem.* **1999**, *76*, 350-354.

Benzie, I.F.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of ‘‘antioxidant power’’: The FRAP assay. *Anal. Biochem.*, **1996**, *239*, 70–76.

Chalker-Scott, L. Environmental significance of anthocyanins in plant stress responses. *Photochem. Photobiol.* **1999**, *70*, 1-9.

Crozier, A.; Jaganath, I.B.; Clifford, M.N. *Plant Secondary Metabolites: Occurrence, Structure and the Role in the Human Diet*. Blackwell Publishing: Oxford, UK, **2006**; pp. 1-372.

Cuevas, E.; Hillebrand, S.; Antezana, A.; Winterhalter, P. Soluble and bound phenolic compounds in different bolivian purple corn (*Zea mays* L.) cultivars. *J. Agric. Food Chem.* **2011**, *59*, 7068-7074.

Danesi, F.; Elementi, S.; Neri, R.; Maranesi, M.; D’Antuono, L. F.; Bordoni, A. Effect of cultivar on the protection of cardiomyocytes from oxidative stress by essential oils and aqueous extracts of basil (*Ocimum basilicum* L.). *J. Agric. Food Chem*. **2008**, *56*, 9911-9917.

Darrah, H.H. Investigation of the cultivars of the basils (Ocimum). *Econ. Bot.* **1974**, *28*, 63-67.

Flanigan, P.M.; Niemeyer, E.D. Effect of cultivar on phenolic levels, anthocyanin composition, and antioxidant properties in purple basil (*Ocimum basilicum* L.). *Food Chem.* **2014**, *164*, 518-526.

Gachovska, T.; Cassada, D.; Subbiah, J.; Hanna, M.; Thippareddi, H.; Snow, D. Enhanced anthocyanin extraction from red cabbage using pulsed electric field processing. *Food Sci.* **2010**, *59*, 143-150

Gould, K.S. Nature’s swiss army knife: the diverse protective roles of anthocyanins in leaves. *J. Biomed. Biotechnol.* **2004**, *5,* 314-320.

Griffiths, L.A. On the distribution of gentisic acid in green plants. *J. Exp. Bot.* **1959**, *10*, 437-442.

He, J.; Giusti, M.M. Anthocyanins: Natural colorants with health-promoting properties. *Annu. Rev. Food Sci. Technol.* **2010**, *1*, 163-187.

Herms, D.A.; Mattson, W.T. The dilemma of plants: to grow or defend. *Q. Rev. Biol.* **1992**, *67*, 283-335.

Hinneburg, I.; Damien, H.J.; Hiltunen, R. Antioxidant activities of extracts from selected culinary herbs and spices. *Food Chem.* **2006**, *97,* 122-129.

Holton, T.A.; Cornish, E.C. Genetics and biochemistry of anthocyanin biosynthesis. *Plant Cell.* **1995**, *7,* 1071-1083.

Huang, D.; Ou, B.; Prior, R.L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841-1856.

Javanmardi, J.; Khalighi, A.; Kashi, A.; Bais, H.P.; Vivanco, J.M. Chemical characterization of basil (*Ocimum basilicum* L.) found in local accessions and used in traditional medicines in Iran. *J. Agric. Food Chem.* **2002**, *50*, 5878-5883.

Kelsey, N.; Hulick, W.; Winter, A.; Ross, E.; Linseman, D. Neuroprotective effects of anthocyanins on apoptosis induced by mitochondrial oxidative stress. *Nutr. Neurosci.* **2011**, *14,* 249-259.

Kwee, E.M.; Niemeyer, E.D. Variations in phenolic composition and antioxidant properties among 15 basil (*Ocimum basilicum* L.) cultivars. *Food Chem.* **2011**, *128*, 1044-1050.

Lee, J.; Scagel, C.F. Chicoric acid found in basil (*Ocimum basilicum* L.) leaves. *Food Chem.* **2009**, *115,* 650-656.

Makri, O.; Kintzios, S. *Ocimum* sp. (Basil): botany, cultivation, pharmaceutical properties, and biotechnology. *J. Herbs Spices Med. Plants.* **2007**, *13,* 123-150.

Nguyen, P.M; Niemeyer, E.D. Effects of nitrogen fertilization on the phenolic composition and antioxidant properties of basil (*Ocimum basilicum* L.). *J. Ag. Food Chem.* **2008**, *56*, 8685-8691.

Petersen, M.; Simmonds, M.S.J. Rosmarinic acid. *Phytochem*. **2003**, *62*, 121-125.

Phippen, W.B.; Simon, J.E. Anthocyanins in basil (*Ocimum basilicum* L.). *J. Agric. Food Chem.* **1998**, *46*, 1734-1738.

Prior, R.L.; Cao, G.; Martin, A.; Sofic, E.; McEwen, J.; O'Brien, C.; Lischner, N.; Ehlenfeldt, M.; Kalt, E.; Krewer, G.; Mainland, C.M. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. *J. Agric. Food Chem.* **1998**, *46,* 2686-2693.

Proestos, C.; Chorianopoulos, N.; Nychas, G.-J. E.; Komaitis, M. RP-HPLC Analysis of the phenolic compounds of plant extracts. Investigation of their antioxidant capacity and their antimicrobial activity. *J. Agric. Food Chem.* **2005**, *53*, 1190-1195.

Singleton, V.L.; Rossi, J.A. Jr. Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. *Am. J. Enol. Vitic*., **1965**, *16*, 144–158.

Stamp, N. Out of the quagmire of plant defense hypotheses. *Q. Rev. Biol*. **2003**, *78*, 23-55.

Tabart, J.; Kevers, C.; Pincemail, J.; Defraigne, J.; Dommes, J. Antioxidant capacity of black currant varies with organ, season, and cultivar. *J. Agric. Food Chem.* **2006**, *54*, 6271-6276.

Werker, E; Putievsky, E.; Ravid, U.; Dudai, N.; Katzir, I. Glandular hairs and essential oils in developing leaves of *Ocimum basilicum* L. (Lamiaceae). *Ann. Bot.* **1993**, *71*, 43-50.

Wrolstad, R.L.; Durst, R.W.; Giusti, M.M.; Rodriguez-Saona, L.E. Analysis of anthocyanins in nutraceuticals. In *Quality Management of Nutraceuticals*. Ho, C.; Zheng, Q.Y, Eds.; American Chemical Society: Washington, D.C., **2001**; pp. 42-62.

Zgórka, G.; Glowniak, K. Variation of free phenolic acids in medicinal plants belonging to the *Lamiaceae* family. *J. Pharm. Biomed. Anal.* **2001**, *26,* 79-87.

Tables and Figures

Table 1. Average total phenolic concentrations (±SD) in gallic acid equivalents (GAE, mg/g DW). Average concentrations with the same letter are not statistically different.

	days after germination											
	9		16		23		35		49		average	
cultivar	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Purple Ruffles	3.43	0.35	4.16	1.37	3.70	0.62	18.03	3.71	12.78	3.74	8.42	1.96
Rosie	3.30	0.19	4.35	0.26	4.99	0.47	20.08	3.38	14.76	1.60	9.50	1.18
Sweet Petra Dark Red	2.43	0.15	5.14	1.15	4.78	0.98	19.77	1.85	11.82	1.02	8.79	1.03
average	3.05*a*	0.23	4.55 *a*	0.93	4.49 *a*	0.69	19.29 *b*	2.98	13.12 *c*	2.12		

Table 2. Growth analysis of basil plants, measured in terms of average above-ground height (cm), above-ground mass (g), and total leaf mass (g) at various levels of maturity. Average growth measurements for a specific level of maturity are denoted as x_x. Average growth per day since the previous level of maturity is denoted as "growth rate."

days after germination									
9		16		23		35		49	
x_1	growth rate	x_2	growth rate	x_3	growth rate	x_4	growth rate	x_5	growth rate
above-ground height (cm)									
3.622	0.402	5.073	0.207	10.753	0.811	24.033	1.107	34.727	0.764
above-ground mass (g)									
0.087	0.010	0.500	0.059	3.207	0.387	16.540	1.111	29.733	0.942
total leaf mass (g)									
0.078	0.009	0.360	0.040	2.324	0.281	10.737	0.701	19.247	0.608

Table 3. Average individual phenolic acid concentrations (±SD), reported in mg/g DW. Average concentrations with the same letter for a single phenolic acid are not statistically different.

	days after germination											
	9		16		23		35		49		average	
cultivar	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
caffeic acid												
Purple Ruffles	0.03	0.00	0.07	0.02	0.06	0.01	0.24	0.04	0.21	0.05	0.12	0.02
Rosie	0.03	0.00	0.05	0.01	0.06	0.02	0.20	0.02	0.23	0.01	0.11	0.01
Sweet Petra Dark Red	0.04	0.01	0.10	0.02	0.10	0.02	0.22	0.04	0.16	0.05	0.12	0.03
average	0.03 *a*	0.00	0.07 *b*	0.02	0.07 *b*	0.02	0.22 *c*	0.03	0.20 *c*	0.04		
p-coumaric acid												
Purple Ruffles	0.02	0.02	0.08	0.02	0.06	0.02	0.58	0.09	0.19	0.06	0.19 *e*	0.04
Rosie	0.02	0.01	0.04	0.02	0.04	0.02	0.30	0.09	0.10	0.01	0.10 *d*	0.03
Sweet Petra Dark Red	0.02	0.01	0.06	0.02	0.06	0.03	0.48	0.07	0.13	0.01	0.15 *e*	0.03
average	0.02 *a*	0.01	0.06 *b*	0.02	0.05 *b*	0.02	0.45 *c*	0.08	0.14 *d*	0.03		
caftaric acid												
Purple Ruffles	0.08	0.01	0.13	0.04	0.08	0.03	0.74	0.27	0.69	0.20	0.34	0.11
Rosie	0.11	0.00	0.08	0.01	0.07	0.02	1.04	0.16	1.14	0.14	0.49	0.07
Sweet Petra Dark Red	0.07	0.02	0.10	0.03	0.08	0.03	0.78	0.24	0.68	0.11	0.34	0.09
average	0.05 *a*	0.01	0.08 *a*	0.02	0.06 *a*	0.02	0.62 *b*	0.14	0.44 *b*	0.08		
gentisic acid												
Purple Ruffles	1.01	0.19	1.43	0.55	1.46	0.71	1.64	0.40	1.24	0.33	1.36	0.44
Rosie	0.85	0.06	1.13	0.58	0.85	0.04	2.07	0.78	1.45	0.89	1.27	0.47
Sweet Petra Dark Red	0.30	0.07	0.52	0.19	2.30	0.31	2.61	0.96	0.95	0.07	1.34	0.32
average	0.72 *b*	0.11	1.03 *a*	0.44	1.54 *b*	0.35	2.11 *b*	0.71	1.21 *b*	0.43		
chicoric acid												
Purple Ruffles	0.14	0.09	0.28	0.06	0.37	0.15	4.52	1.02	1.67	0.52	1.40	0.37
Rosie	0.13	0.03	0.25	0.10	0.24	0.08	2.48	0.32	1.40	0.27	0.90	0.16
Sweet Petra Dark Red	0.12	0.07	0.47	0.10	0.44	0.21	3.66	0.40	1.68	0.30	1.27	0.22
average	0.13 *a*	0.06	0.33 *b*	0.09	0.35 *b*	0.15	3.55 *c*	0.58	1.58 *d*	0.36		
rosmarinic acid												
Purple Ruffles	1.27	0.21	3.90	1.34	3.43	0.79	22.77	7.06	4.22	3.10	7.12	2.50
Rosie	1.86	0.31	3.28	1.16	3.31	0.74	17.61	3.41	6.55	2.29	6.52	1.58
Sweet Petra Dark Red	0.79	0.25	2.39	0.52	2.17	0.66	22.36	4.11	4.81	3.07	6.50	1.72
average	0.63 *a*	0.15	1.56 *b*	0.48	1.47 *b*	0.40	10.99 *c*	2.41	3.13 *b*	1.42		

Table 4. Average individual anthocyanin concentrations (±SD) in kuromanin equivalents (KE, mg/g DW). Average concentrations with the same letter for a single anthocyanin are not statistically different.

	days after germination											
	9		16		23		35		49		average	
cultivar	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
anthocyanin A												
Purple Ruffles	0.22	0.03	0.41	0.13	0.53	0.15	0.82	0.23	0.45	0.14	0.49 *d*	0.14
Rosie	0.38	0.06	0.47	0.11	0.44	0.06	0.73	0.25	0.31	0.09	0.47 *d*	0.11
Sweet Petra Dark Red	0.13	0.02	0.32	0.03	0.22	0.02	0.60	0.10	0.46	0.10	0.35 *e*	0.05
average	0.24 *a*	0.04	0.40 *a*	0.09	0.40 *a*	0.08	0.72 *b*	0.19	0.41 *a*	0.11		
anthocyanin B												
Purple Ruffles	0.08	0.03	0.25	0.08	0.31	0.08	0.44	0.11	0.16	0.04	0.25 *d*	0.07
Rosie	0.21	0.04	0.29	0.07	0.17	0.12	0.32	0.11	0.06	0.01	0.21 *d*	0.07
Sweet Petra Dark Red	0.05	0.01	0.20	0.07	0.26	0.03	0.28	0.06	0.12	0.02	0.18 *e*	0.04
average	0.11 *a*	0.03	0.25 *b*	0.07	0.25 *b*	0.08	0.35 *c*	0.09	0.11 *a*	0.02		
anthocyanin C												
Purple Ruffles	0.89	0.07	1.61	0.33	2.02	0.55	1.80	0.57	1.18	0.47	1.50 *d*	0.40
Rosie	0.90	0.14	1.22	0.25	1.18	0.17	1.05	0.39	0.62	0.29	0.99 *e*	0.25
Sweet Petra Dark Red	0.41	0.02	1.26	0.33	1.03	0.49	1.14	0.29	0.83	0.20	0.93 *e*	0.27
average	0.73 *a*	0.08	1.36 *b*	0.30	1.41 *b*	0.40	1.33 *bc*	0.42	0.88 *c*	0.32		
anthocyanin D												
Purple Ruffles	0.37	0.04	0.98	0.25	1.18	0.31	0.92	0.25	0.35	0.13	0.76 *d*	0.20
Rosie	0.58	0.12	0.91	0.23	0.84	0.12	0.51	0.17	0.10	0.01	0.59 *e*	0.13
Sweet Petra Dark Red	0.21	0.01	0.78	0.22	0.59	0.32	0.51	0.20	0.21	0.05	0.46 *f*	0.16
average	0.39 *a*	0.06	0.89 *b*	0.23	0.87 *bc*	0.25	0.65 *c*	0.21	0.22 *a*	0.06		

Table 5. Average antioxidant capacities (±SD) measured in trolox equivalents (TEAC, mmol/g DW). Average antioxidant capacities with the same letter for a single type of extract are not statistically different.

	days after germination											
	9		16		23		35		49		average	
cultivar	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
anthocyanins												
Purple Ruffles	4.32	0.33	7.02	0.99	8.07	1.95	23.22	7.79	20.04	7.54	13.25	3.72
Rosie	5.14	0.85	7.38	0.92	7.57	0.73	28.73	12.80	27.72	3.76	15.09	3.81
Sweet Petra Dark Red	3.50	0.40	10.75	4.32	7.06	0.84	26.22	0.67	24.21	3.08	14.81	1.86
average	4.32 *a*	0.53	8.39 *b*	2.08	7.60 *b*	1.17	26.06 *c*	7.09	23.27 *c*	4.79		
phenolic acids												
Purple Ruffles	2.18	0.33	3.90	1.28	3.54	0.56	23.39	8.49	11.72	4.55	9.53 *d*	3.04
Rosie	2.65	0.73	5.90	1.82	6.80	1.49	34.50	16.30	16.77	6.31	14.01 *e*	5.33
Sweet Petra Dark Red	2.63	0.71	5.23	0.56	6.83	1.47	34.39	13.41	16.46	6.44	13.79 *e*	4.52
average	2.48 *a*	0.59	5.00 *b*	1.22	5.72 *ab*	1.17	30.76 *c*	12.73	14.39 *d*	5.77		

Figure 1. Average total anthocyanin concentrations (±SD) reported in anthocyanin equivalents (AE, mg/g DW) for Purple Ruffles (PR), Rosie, and Sweet Petra Dark Red (SPDR). Average concentrations for each harvest date are depicted below the x-axis. Average concentrations with the same letter are not statistically different.

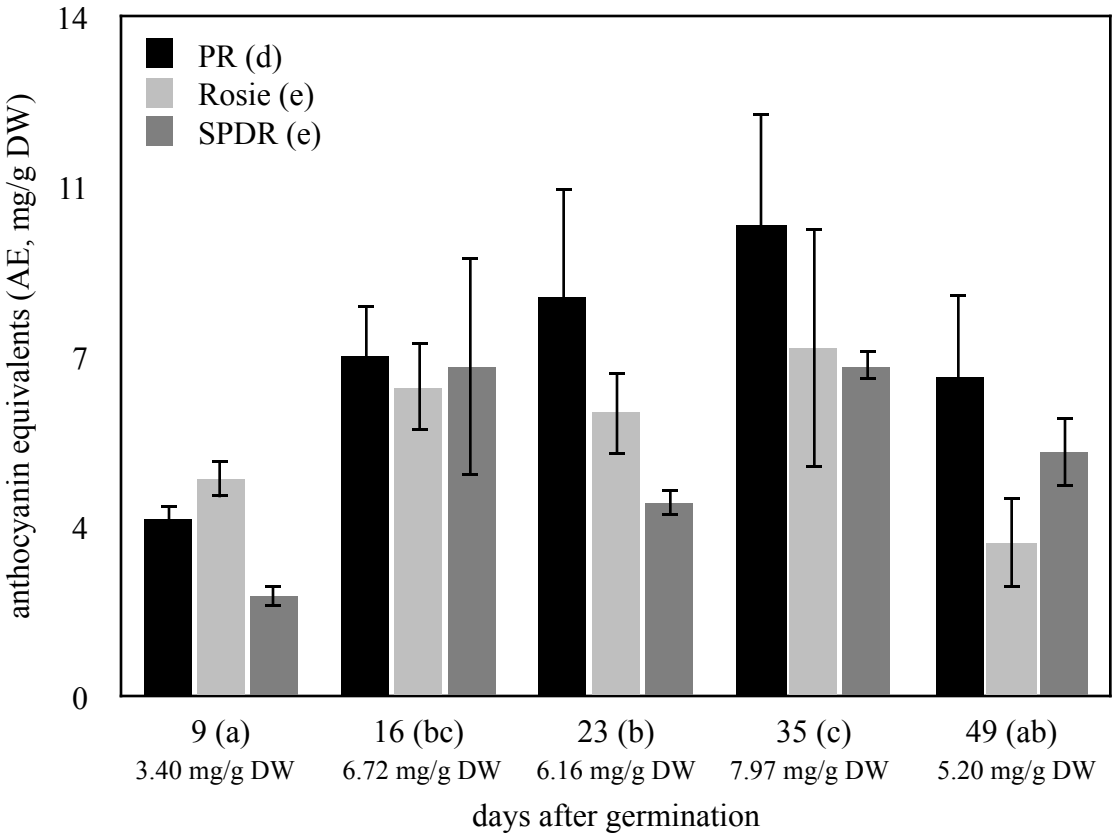


Figure 2. Dendrogram plot displaying the clustering of the five levels of basil maturity in this study based on their concentrations of (A) individual phenolic acids and (B) anthocyanins A-D.

