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

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ABSTRACT

We have determined the effect of plant maturity on total and individual anthocyanin concentrations, phenolic acid content, and antioxidant capacities in three purple basil (*Ocimum basilicum* L.) cultivars. Plant maturity significantly influenced total phenolic content (with values ranging from 3.30 to 20.08 mg/g dry weight, DW) and concentrations of individual phenolics, including caftaric (0.08 to 0.85 mg/g DW), chicoric (0.13 to 3.55 mg/g DW), and rosmarinic (1.31 to 21.31 mg/g DW) acids. Cultivar and plant maturity significantly affected the total anthocyanin content (2.07 to 9.72 mg/g DW) and the concentrations of the four most abundant individual anthocyanins. Plant maturity also had a significant effect on measured FRAP (ferric reducing antioxidant power) reducing capacities (3.50 to 28.73 mmol/100 g DW), and phenolic acid concentrations were found to have an important influence on basil 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.

Keywords: *Ocimum basilicum* L.; basil; food analysis; high-performance liquid chromatography; phenolic acids; anthocyanins; food composition; antioxidants; Lamiaceae family

1. Introduction

Plants synthesize an enormous variety of phenolic compounds to fulfill their primary needs, such as photosynthesis, growth, and development, as well as to aid in secondary functions, including attraction of pollinators 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 (Zgórk & Glowniak, 2001). Basil is rich in rosmarinic, caftaric, and chicoric acids (Flanigan & Niemeyer, 2014; Kwee & Niemeyer, 2011; Lee & Scagel, 2009; Zgórk & Glowniak, 2001;) and possesses antioxidant properties (Hinneburg, Damien, & Hiltunen, 2006) which may confer an array of health benefits within the human diet. Purple varieties of basil also contain anthocyanins, a group of red-blue plant pigments which belong to one of the largest classes of secondary metabolites known as flavonoids. Anthocyanins serve a variety of functions in plants – from protecting against UV radiation to attracting pollinating insects (Gould, 2004) – while also providing numerous health-promoting benefits to humans including anti-inflammatory, anticarcinogenic, and neuroprotective effects (He & Giusti, 2010; Kelsey, Hulick, Winter, Ross & Liseman, 2011).

It is well established that many plants use similar pathways to synthesize secondary metabolites, but the quantities and types of phenolic compounds and anthocyanins produced by a plant are greatly affected by genetic factors (Holton & Cornish, 1995), environmental conditions (Poiroux-Gonard, Bidel, Fanciullino, Gautier; Lauri-Lopez & Urban, 2010) and other factors such as the level of plant maturity (Parr & Bolwell, 2000; Schreiner, 2005). Plant maturity in particular has been reported to cause significant variations in anthocyanin content, phenolic concentrations, and antioxidant properties within blueberries and black currants (Prior et al., 1998; Tabart, Kevers, Pincemail, Defraigne & Dommes, 2006). In purple basil, Phippen and

Simon (1998) found that anthocyanin composition varied in the Purple Ruffles cultivar as the plant matured, with total anthocyanin concentrations increasing prior to flowering then subsequently decreasing.

Little is currently known, however, about the effect of plant maturity on phenolic acid concentrations in purple basil or the contribution of anthocyanins and phenolic acids to basil's antioxidant properties as the herb grows. Basil synthesizes a range of secondary metabolites with potential disease-preventing properties, and determining optimal levels of these compounds may enhance the herb's nutritional and medicinal value. Therefore, the purpose of the current study is to determine how the growth of basil from seedling to mature plant influences total and individual anthocyanin and phenolic acid concentrations as well as measured antioxidant capacities within three purple cultivars.

2. Materials and methods

2.1. Chemicals and reagents

The analytical standards 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 purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol were high-performance liquid chromatography (HPLC) grade and were obtained from Pharmco-AAPER (Brookfield, CT, USA) 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, USA) and Fisher Scientific (Fair Lawn, NJ, USA), respectively. Sodium carbonate was purchased from

EMD Chemicals, Inc. (Gibbstown, NJ, USA), and Folin-Ciocalteu reagent was obtained from VWR International (West Chester, PA, USA).

2.2. Plant materials

Three 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 (*O. basilicum* 'Rosie') from Richter's Herbs (Goodwood, Ontario), Purple Ruffles (*O. basilicum* 'Purple Ruffles') from Johnny's Selected Seeds Company (Winslow, ME, USA), and Sweet Petra Dark Red (*O. basilicum* 'Sweet Petra Dark Red') from W. Atlee Burpee & Co. (Warminster, PA, USA). 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, USA) on May 26, 2011. Plants were grown under natural light and temperature conditions 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).

2.3. Plant harvesting, growing conditions, and growth analysis

Basil leaves from each cultivar were harvested on five unique dates: 9, 16, 23, 35, and 49 DAG. Harvest dates were selected to focus on early stages of plant growth (before 60 DAG) based on a previous study of the Purple Ruffles basil cultivar showing that the greatest changes in anthocyanin content occurred within this time frame (Phippen & Simon, 1998). For the first harvest 9 DAG, collected samples consisted of combined seedlings in order to obtain enough foliar mass for later analysis ($n = 9$; 3 replicates for each cultivar). Remaining healthy seedlings were transferred to one-gallon pots containing mesh bags filled with Ferti-lome Ready-to-Use Ultimate Potting Mix (Cheek Garden Products, Austin, TX, USA). Fifty-seven pots were organized in a randomized block design with 5 replicates per cultivar for three of the remaining

harvest dates (16, 23 and 35 DAG) and 4 replicates per cultivar for the final harvest date (49 DAG). Based on this experimental design, 66 independent plant samples were therefore grown for later analysis. Plants remained in the greenhouse (average temperature range: 25.2 - 46.5°C; average humidity range: 26.3 - 88.4%) until the appropriate harvest date. Individual replicates containing leaves obtained from a single plant were harvested, immediately frozen in liquid nitrogen and stored at -80°C until processing.

During harvests, the above-ground height, total leaf mass, and total plant mass were measured for each plant. Average plant height, plant mass, and leaf mass were then calculated 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.

2.4. Basil extract preparation

Phenolic compounds were extracted according to a previous method (Nguyen & Niemeyer, 2008). 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 then the dried basil was incubated in 1.0 mL of 80% aqueous methanol and 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 μ m nylon filter, and stored at -80°C until further analysis. Anthocyanins were extracted by incubating a known mass of 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 nylon filter and stored at -80°C until analysis.

2.5. 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, USA), 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 previously developed method (Abdel-Aal & 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 cyanidin 3-*O*-glucoside 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.

2.6. Antioxidant reducing capacity determination

Antioxidant reducing capacities of acidified 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 in 40 mM HCl. For analysis, 25 μ L of extract was incubated with 250 μ L of FRAP reagent in a 96-well microplate and shaken at room temperature for 8 minutes. Absorbance of each extract (A_{sample}) was measured in triplicate at 593 nm and compared to a control (A_{control}), consisting of 25 μ L of acidified or 80% methanol and 250 μ L of FRAP reagent. The average difference in absorbance ($A = A_{\text{sample}} - A_{\text{control}}$) of each sample was compared to a calibration curve of trolox in acidified methanol, ranging from 20.00 to 1200.00 μ M. If initial analysis indicated that the sample absorbance was greater than the highest concentration trolox standard, the sample was diluted with acidified methanol and re-analyzed. Antioxidant reducing capacities were expressed as trolox equivalent antioxidant capacities (TEAC) in mmol/100 g DW.

2.7. Quantification of individual phenolic acids and anthocyanins

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, USA) equipped with a Phenomenex Kinetex (Torrance, CA, USA) C-18 column (2.6 μ m, 4.6 mm x 100 mm) and a UV absorbance detector. Eluent A was 3% methanol and 1% formic acid in water and eluent B was 0.1% formic acid in acetonitrile. The sample injection size was 10 μ L, the flow rate was 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. For some extracts with high rosmarinic acid concentrations, dilutions with 80% methanol were necessary before analysis. Detection occurred at 280 nm, and phenolic acids were identified in the samples by comparing their chromatographic retention times against analytical standards (for typical standard and sample chromatograms, see Supplementary Material, Figures 1 and 2). 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).

Quantification of individual anthocyanins was accomplished with a tri-pump Shimadzu HPLC system (Columbia, MD, USA) equipped with a 50 μ L injection loop, Phenomenex Luna C-18 column (5 μ m, 4.6 mm x 250 mm), and diode-array detector. 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 was used: 16% B, 0 min; 20% B, 10 min; 23% B, 35 min; 50% B, 50 min; 16% B, 52-62 min. If initial analysis indicated the sample had low anthocyanin concentrations, the sample was concentrated by evaporating the solvent using a gentle stream of nitrogen gas then reconstituted using a smaller volume of acidified methanol. The four anthocyanins found in highest abundance within all basil cultivars were quantified by integrating chromatographic peak areas and comparing them to an external calibration curve

prepared from cyanidin 3-*O*-glucoside chloride in acidified methanol, ranging from 0.31 to 25.00 mg/L (for a typical sample chromatogram, see Supplementary Material, Figure 3). Final individual anthocyanin concentrations were reported as anthocyanin equivalents (AE) in mg/g DW.

2.8. Structural characterization of individual anthocyanins

Initial structural characterization of the four anthocyanins found in highest abundance was achieved using an Agilent 1260 Infinity HPLC system with a Gemini C-18 column (3.5 μ m, 2.1 mm x 50 mm) interfaced with an Agilent 6530 Accurate-Mass QTOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). Gradient separation conditions were the same as those described above. 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 optimized for the analysis. Mass spectra were collected from 100-3200 m/z with an acquisition rate of 1.00 spectra/sec. The four highest concentration anthocyanins were identified according to elution order and molecular ion as follows: **1**, retention time, t_r = 29 min, $[M+H]^+ = m/z$ 1081.3; **2**, retention time, t_r = 34 min, $[M+H]^+ = m/z$ 1167.3; **3**, retention time, t_r = 38 min, $[M+H]^+ = m/z$ 1065.3; **4**, retention time, t_r = 46 min, $[M+H]^+ = m/z$ 1151.3.

Further structural characterization of the four anthocyanins was accomplished using a Dionex Ultimate 3000 RSLC nanoLC system (Thermo Scientific, Waltham, MA, USA) equipped with a nanotip column (3.5 μ m, 15 cm x 100 μ m; New Objective, Woburn, MA, USA) packed in-house with C18 media and interfaced with a Velos Pro dual-pressure linear ion trap mass spectrometer (Thermo Scientific, San Jose, CA, USA). Solvent from an aliquot of basil extract was first dried using vacuum centrifugation and the sample was then reconstituted in

0.1% aqueous formic acid prior to injection. Eluent A consisted of 2% acetonitrile and 0.1% formic acid in water and eluent B was 100% acetonitrile. A flow rate of 0.3 $\mu\text{L}/\text{min}$ with a linear gradient of 0-50% B was used over 50 minutes and the injection volume was 1 μL . Data-independent MS^n analyses were performed on anthocyanin precursor ions using positive electrospray ionization (ESI) with ionization conditions of 2.0 kV for spray voltage and 275°C for capillary temperature. Mass spectra were collected from m/z 250-2000, and three microscans per scan with a maximum injection time of 10 ms were averaged. Tentative structural identities of the anthocyanins were elucidated by comparing their MS^2 and MS^3 fragmentation patterns to basil anthocyanin MS^n data described in the literature (Luna, Bekhradi, Ferreres, Jordán, Delshad & Gil, 2015).

2.9. Statistical analyses

All statistical analyses were performed using IBM SPSS Statistics, version 22.0 (SPSS Inc., Chicago, IL, USA). All data are reported as the mean $\pm$ 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.

3. Results and discussion

3.1. 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, our total phenolic concentrations are similar to those previously reported for purple basil cultivars by Flanigan and Niemeyer (2014): 22.01, 15.12, and 13.09 mg/g DW for Sweet Petra, Rosie, and Purple Ruffles cultivars, respectively.

Cultivar did not have a significant effect on the total phenolic concentration. However, plant maturity at the time of harvest significantly influenced 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 content than basil at 35 DAG ($p = 0.003$). To investigate this relationship further, basil growth rates were compared between different plant maturities (see Supplementary Material, 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 metabolites – particularly low molecular weight phenolic compounds – are sometimes at highest concentrations in early stages of growth or only synthesized at young stages of plant development when leaves are most in need of defense from herbivores (Herms & Mattson, 1992; Parr & Bolwell, 2000). For example, galloylglucoses are hydrolysable tannins that reach their highest levels in young white birch leaves, with concentrations of these compounds decreasing in leaf tissue due to dilution as growth continues and leaf mass subsequently increases (Salminen, Ossipov, Haukioja, & Pihlaja, 2001). In the purple basil cultivars in this study, phenolic content may therefore increase in

young leaves as a deterrent to herbivores but following a period of rapid plant growth, a decrease in phenolic concentration occurs due to increasing leaf mass. 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).

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 2. 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 that are commonly found in herbs, including the Lamiaceae family (Zgórk & Glowniak, 2001). Caffeic acid was the least abundant phenolic acid included in our study, with average concentrations ranging from 0.03 to 0.22 mg/g DW at 9 and 35 DAG, respectively. The low levels we observed were similar to those reported in the literature for other purple basil varieties (Flanigan & Niemeyer, 2014) as well as green Iranian basil, which was found to have caffeic acid concentrations no greater than 0.003 mg/g DW (Javanmardi, Khalighi, Kashi, Bais, & Vivanco, 2002). Iranian basil was also reported to have *p*-coumaric acid concentrations less than 0.35 mg/g DW in the leaves and flowers of several accessions (Javanmardi et al., 2002). Our concentrations for purple basil in this study were within the same range, with average values of 0.02 (9 DAG) to 0.45 mg/g DW (35 DAG). Cultivar had a significant effect on *p*-coumaric acid concentrations ($p = 0.01$), with Rosie having significantly lower levels (0.12 mg/g DW) than Purple Ruffles (0.22 mg/g DW; $p = 0.012$) and Sweet Petra Dark Red (0.19 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 and only 0.004 mg/g FW for Purple Petra. In our study, average caftaric acid concentrations ranged from 0.08 (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 basil cultivars, where the highest concentration of caftaric acid was 0.12 mg/g DW for the cultivar Amethyst Improved (Flanigan & Niemeyer, 2014). Although our caftaric acid concentrations are also higher than those found by Lee and Scagel (2009) for a purple basil cultivar, it is difficult for us to make comparisons with their findings because they analyzed fresh plant tissue (we analyzed dried) and water content can result in lower reported phenolic concentrations.

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 (Flanigan & Niemeyer, 2014; Javanmardi et al., 2002; Proestos, Chorianopoulos, Nychas, & Komaitis, 2005). 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 (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 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 and its presence in herbs is associated with a multitude of health benefits (Crozier et al., 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 content, with basil at 9 DAG having lower concentrations than all other levels of maturity ($p = 0.008$), and 35 DAG having greater concentrations 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 contents 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 concentrations, 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).

3.2. 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 (2014), who observed total anthocyanin contents ranging from 7.55 to 16.6 mg/g DW for the cultivars Rubin and Purple Ruffles (Richter's Herbs), respectively. Although all basil from their study was harvested at 39 DAG, we did not observe concentrations as high as Flanigan and Niemeyer (2014), 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 (1998), who reported Purple Ruffles from Johnny's Selected Seeds had an average concentration of 0.16 mg/g FW. Phippen and Simon (1998) analyzed fresh plant tissue, whereas in our study anthocyanins were extracted from dried leaves. Additionally, Phippen and Simon (1998) extracted from stems and flowers, which may underestimate total anthocyanin concentrations since accumulation in the leaves is the largest contributor to basil's purple color (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 concentrations 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 (1998) observation that large-leaf varieties of purple basil, such as Purple Ruffles, contain greater anthocyanin concentrations than other varieties. Additionally, Flanigan and Niemeyer (2014) found Purple Ruffles to have significantly higher anthocyanin content than one other purple cultivar, Rubin.

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 concentrations than basil at 9 ($p < 0.01$), 23 ($p = 0.029$), and 49 DAG ($p < 0.01$). Our anthocyanin values are much higher than those reported by Phippen and Simon (1998) in their growth study of Burpee's Purple Ruffles. Their basil 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 (1998) study. It is interesting to note that basil harvested at 49 DAG had significantly lower total anthocyanin concentrations than basil harvested at 35 DAG in our study, because in Phippen and Simon's (1998) study, 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 or increased leaf mass of the basil plants between 35 and 49

DAG may explain the decrease we observed 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 concentrations ($p = 0.048$). This result indicates that plant maturity and cultivar have a unique combined effect on total anthocyanin contents.

Mass spectral analysis was used to structurally characterize the four most abundant individual anthocyanins found in the basil extracts (see Supplementary Material, Table 1) and based on diagnostic MS^n fragment ions and the mass spectral characterization of purple Iranian basil completed by Luna et al. (2015), the four most abundant anthocyanins were tentatively assigned as: **1**, cyanidin-3-O-(6-*p*-coumaroyl-6'-caffeoyl)sophoroside-5-O-glucoside; **2**, cyanidin-3-O-(6-*p*-coumaroyl-X-malonyl-6'-caffeoyl)sophoroside-5-O-glucoside; **3**, cyanidin-3-O-(6,6'-di-*p*-coumaroyl)sophoroside-5-O-glucoside; and **4**, cyanidin-3-O-(6,6'-di-*p*-coumaroyl-X-malonyl)sophoroside-5-O-glucoside (where X denotes that the position of the malonyl substituent could not be unambiguously identified). Average concentrations for these anthocyanins, measured in anthocyanin equivalents (AE), are displayed in Table 3. These four most abundant anthocyanins were detected in all cultivars at all levels of maturity that were studied. Anthocyanin **3**, a cyanidin-based *p*-coumaroyl glucoside with m/z 1065, 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 **2**, a cyanidin-based *p*-coumaroyl and malonyl glucoside with m/z 1167, 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 (1998) 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. For

instance, the compound that we identified as anthocyanin **3** was present in the highest quantity in their study, and the chromatographic peak that we denote as anthocyanin **2** was present in lowest quantity in Phippen and Simon's (1998) study.

Cultivar had a significant effect on the content of individual anthocyanins, with Purple Ruffles producing higher concentrations of individual anthocyanins than Sweet Petra Dark Red for all anthocyanins (**1**, $p = 0.001$; **2**, $p < 0.01$; **3**, $p < 0.01$; **4**, $p < 0.01$), and higher concentrations than Rosie for anthocyanins **3** ($p < 0.01$) and **4**, a cyanidin-based *p*-coumaryl and malonyl glucoside with m/z 1151 ($p = 0.024$) only. Plant maturity at the time of harvest also significantly affected the concentrations of the four most abundant anthocyanins, with basil harvested at 35 DAG containing greater individual anthocyanin concentrations than basil that had been harvested at 9 DAG (**1**, $p < 0.01$; **2**, $p < 0.01$; **3**, $p = 0.003$; **4**, $p = 0.007$). 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 **3**, and lower than 16, 23, and 35 DAG for anthocyanins **2** (16 DAG, $p = 0.001$; 23 DAG, $p = 0.001$; 35 DAG, $p < 0.01$) and **4** (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, $p < 0.01$). A significant interaction between cultivar and harvest date was found for anthocyanins **2** ($p = 0.005$) and **4** ($p = 0.021$), indicating that the concentrations of these anthocyanins were impacted differently by harvest date among the cultivars studied. For example, anthocyanin **4** concentrations increased until 16 DAG then declined for the Rosie and Sweet Petra Dark Red cultivars. For the Purple Ruffles cultivar, however, concentrations of anthocyanin **4** reached their maximum at 23 DAG before subsequently declining at later harvest dates.

3.3. 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 concentrations 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 concentrations of all six phenolic acids. The third cluster contained basil harvested at 49 DAG and had high concentrations of caftaric and caffeic acids, moderate amounts of chicoric acid, and low concentrations of gentisic, *p*-coumaric, and rosmarinic acids.

In grouping the maturities based on similarities in individual anthocyanin composition (Figure 2B), the largest cluster contained basil harvested at 16 and 23 DAG, which was characterized by moderate concentrations of anthocyanin **1**, a cyanidin-based *p*-coumaryl glucoside with *m/z* 1081, and **2** and high amounts of anthocyanins **3** and **4**. Basil harvested at 9 DAG was contained in its own group and had low concentrations of the individual anthocyanins. A third cluster contained basil harvested at 35 DAG, which had high concentrations of the individual anthocyanins, and the fourth cluster contained basil harvested at 49 DAG, which contained moderate amounts of anthocyanins **1** and **3** but low concentrations of anthocyanins **2** and **4**.

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 concentrations of all anthocyanins and all phenolic acids. Basil at 49 DAG, however, was placed in a cluster that had low to moderate concentrations of individual anthocyanins, 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 concentrations of individual anthocyanins, but low amounts of all six phenolic acids. This, too, implies that at certain stages of growth, basil may experience a trade-off in metabolite production, which has been previously observed when anthocyanin and phenolic acid profiles were compared in mature purple basil cultivars (Flanigan & Niemeyer, 2014). 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).

3.4. Antioxidant reducing capacities

Extractability of phenolic acids and anthocyanins is greatly influenced by factors such as temperature, pH, and the composition of extraction solvents. It is advantageous to perform anthocyanin extraction using acidified solvents (Gachovska, Cassada, Subbiah, Hanna, Thippareddi, & Snow, 2010; Wrolstad, Durst, Giusti, & Rodriguez-Soana, 2001) since 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 et al., 2001). Although extraction methods are optimized to maximize the yield of a variety of compounds – including the analyte(s) of interest – extractions are not entirely selective processes. Phenolic compounds and anthocyanins, for example, are both extractable in acidified methanol. The FRAP antioxidant

capacities of acidified methanolic extracts are therefore likely to measure the antioxidant properties of anthocyanins as well as other phenolic acids which are co-extracted.

Table 4 presents the average antioxidant reducing capacities of acidified 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 reducing capacities 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 reducing capacities, the maturity of basil at harvest did have a significant influence ($p < 0.01$). Basil at 9 DAG had significantly lower antioxidant reducing 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 reducing 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 reducing capacities did not differ between basil harvested at 35 and 49 DAG. The FRAP antioxidant values of basil at 35 DAG are comparative to those reported by Flanigan and Niemeyer (2014) whose antioxidant reducing capacities ranged from 24.6 (Johnny's Purple Ruffles) to 38.4 mmol/100 g DW (Purple Delight).

Plotting the average total anthocyanin concentration against the FRAP antioxidant reducing capacity (data not shown), resulted in a very low level of correlation ($y = 1.88x + 2.61$ by linear least squares analysis; $R^2 = 0.1876$), suggesting that total anthocyanin content does not strongly affect the measured antioxidant capacity. For example, Rosie basil at 49 DAG had the second lowest total anthocyanin concentration, yet contained the second highest FRAP antioxidant reducing capacity. Because phenolic acids may be co-extracted with anthocyanins in

acidified methanolic extracts, the combined influence of anthocyanin and phenolic concentrations on antioxidant reducing capacities was investigated. The sum of the total anthocyanin and phenolic concentrations was plotted against the FRAP antioxidant reducing capacity (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, suggesting the importance of phenolics in influencing the antioxidant reducing capacity of basil relative to anthocyanins.

Examination of hierarchical cluster analysis results in the context of measured antioxidant reducing capacities reveals several interesting relationships. Basil at 9 and 35 DAG were placed in clusters characterized by low and high concentrations of all individual anthocyanins and phenolic acids, respectively, and they also correspondingly had the lowest and highest average antioxidant reducing capacities. Basil at 16 and 23 DAG was characterized as having moderate to high concentrations of individual anthocyanins, and low levels of all phenolic acids with relatively low measured average antioxidant reducing capacities. However, basil at 49 DAG possessed low to moderate concentrations of individual anthocyanins, had high levels of two phenolics, and expressed some of the highest average antioxidant reducing capacities reported in our study. Although total anthocyanin concentrations were poorly correlated with their associated antioxidant reducing capacities, individual anthocyanin and phenolic composition seems to have a greater influence on measured average antioxidant capacities of acidified methanolic extracts. Additionally, these results suggest that total and individual phenolic concentrations are the largest contributors to measured antioxidant reducing capacities. Overall, phenolic concentrations serve as the primary indicators of the antioxidant

properties of basil, whereas antioxidant reducing capacities are less directly dependent on anthocyanin concentrations.

4. 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 reducing capacity of purple basil. Plant maturity significantly influenced the total and individual anthocyanin and phenolic concentrations as well as the measured antioxidant reducing capacities, while cultivar had a significant effect on total and individual anthocyanin concentrations and FRAP 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 in relation to their acidified methanolic antioxidant reducing 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 reducing 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 reducing capacities; the third group (35 DAG), containing high concentrations of all anthocyanins and phenolic acids, had high antioxidant reducing capacities; and the fourth group (49 DAG), with low to moderate concentrations of anthocyanins but high levels of gallic and caffeic acids, had relatively high antioxidant reducing 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 reducing

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.

Supplementary material

Retention times and mass spectral data used in the tentative identification of the four most abundant individual anthocyanins are presented in Supplementary Material Table S1. A growth analysis of basil plants in this study, measured in terms of average above-ground height, above-ground mass, and total leaf mass, is presented at various levels of maturity in Supplementary Material Table S2. Example chromatograms for the phenolic acid standard mixture (Supplementary Material, Figure S1), an 80% aqueous methanolic basil extract (Supplementary Material, Figure S2), and an acidified methanolic basil extract (Supplementary Material, Figure S3) are also provided.

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FIGURE CAPTIONS

Figure 1. Average total anthocyanin concentrations ($\bar{x} \pm$ standard deviation, SD; determined from n = 66 independent samples: 9 DAG, n = 9; 16, 23, and 35 DAG, n = 15; 49 DAG, n = 12) reported in anthocyanin equivalents (AE, mg/g dry weight, DW) for Purple Ruffles, Rosie, and Sweet Petra Dark Red. Average concentrations for each harvest date are reported below the x-axis. Average concentrations with the same letter are not statistically different.

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

Figure 1

Figure 2

Table 1

Average total phenolic concentrations ($\bar{x} \pm$ standard deviation, SD) in gallic acid equivalents (GAE, mg/g dry weight, DW).^{a,b}

cultivar	days after germination											
	9		16		23		35		49		average	
	$\bar{x}$	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	8.33	6.24
Rosie	3.30	0.19	4.35	0.26	4.99	0.47	20.08	3.38	14.76	1.60	9.58	7.22
Sweet Petra Dark Red	2.43	0.15	5.14	1.15	4.78	0.98	19.77	1.85	11.82	1.02	9.10	1.48
average	3.05 a	0.52	4.55 a	1.06	4.49 a	0.89	19.30 b	3.00	11.97 c	2.79		

^a Average concentrations with the same letter are not statistically different.

^b n = 66 independent samples analyzed; 9 DAG, n = 9; 16, 23, and 35 DAG, n = 15; 49 DAG, n = 12.

Table 2

Average individual phenolic acid concentrations ($\bar{x} \pm$ standard deviation, SD), reported in mg/g dry weight, DW.^{a,b}

cultivar	days after germination											
	9		16		23		35		49		average	
	$\bar{x}$	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	0.14	0.09
Rosie	0.03	0.00	0.05	0.01	0.06	0.02	0.20	0.02	0.23	0.01	0.11	0.08
Sweet Petra Dark Red	0.04	0.01	0.10	0.02	0.10	0.02	0.22	0.04	0.16	0.05	0.13	0.07
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		
<i>p</i> -coumaric												
Purple Ruffles	0.02	0.02	0.08	0.02	0.06	0.02	0.58	0.09	0.19	0.06	0.20 e	0.22
Rosie	0.02	0.01	0.04	0.02	0.04	0.02	0.30	0.09	0.10	0.01	0.11 d	0.12
Sweet Petra Dark Red	0.02	0.01	0.06	0.02	0.06	0.03	0.48	0.07	0.13	0.01	0.16 e	0.19
average	0.02 a	0.01	0.06 b	0.02	0.05 b	0.02	0.45 c	0.14	0.15 d	0.06		
caftaric acid												
Purple Ruffles	0.08	0.01	0.13	0.04	0.08	0.03	0.74	0.27	0.69	0.20	0.37	0.34
Rosie	0.11	0.00	0.08	0.01	0.07	0.02	1.04	0.16	1.14	0.14	0.46	0.50
Sweet Petra Dark Red	0.07	0.02	0.10	0.03	0.08	0.03	0.78	0.24	0.68	0.11	0.33	0.34
average	0.08 a	0.02	0.10 a	0.03	0.08 a	0.02	0.85 b	0.25	0.81 b	0.26		
gentisic acid												
Purple Ruffles	1.01	0.19	1.43	0.55	1.46	0.71	1.64	0.40	1.24	0.33	1.41	0.48
Rosie	0.85	0.06	1.13	0.58	0.85	0.04	2.07	0.78	1.45	0.89	1.29	0.71
Sweet Petra Dark Red	0.30	0.07	0.52	0.19	2.30	0.31	2.61	0.96	0.95	0.07	1.53	1.08
average	0.80 a	0.34	1.03 b	0.59	1.54 a	0.74	2.11 a	0.81	1.24 a	0.50		
chicoric acid												
Purple Ruffles	0.14	0.09	0.28	0.06	0.37	0.15	4.52	1.02	1.67	0.52	1.56	1.81
Rosie	0.13	0.03	0.25	0.10	0.24	0.08	2.48	0.32	1.40	0.27	0.93	1.00
Sweet Petra Dark Red	0.12	0.07	0.47	0.10	0.44	0.21	3.66	0.40	1.68	0.30	1.35	1.42
average	0.13 a	0.06	0.34 b	0.13	0.35 b	0.17	3.55 c	1.06	1.60 d	1.42		
rosmarinic acid												
Purple Ruffles	1.27	0.21	3.90	1.34	3.43	0.79	22.77	7.06	4.22	3.10	7.76	8.81
Rosie	1.86	0.31	3.28	1.16	3.31	0.74	17.61	3.41	6.55	2.29	6.96	6.50
Sweet Petra Dark Red	0.79	0.25	2.39	0.52	2.17	0.66	22.36	4.11	4.81	3.07	6.60	9.04
average	1.31 a	0.52	3.19 b	1.12	2.97 b	0.90	21.31 c	5.27	5.30 b	2.69		

^a Average concentrations with the same letter are not statistically different.

^b n = 66 independent samples analyzed; 9 DAG, n = 9; 16, 23, and 35 DAG, n = 15; 49 DAG, n = 12.

Table 3

Average individual anthocyanin concentrations ($\bar{x} \pm$ standard deviation, SD) in anthocyanin equivalents (AE, mg/g dry weight, DW).^{a,b}

cultivar	days after germination											
	9		16		23		35		49		average	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
anthocyanin 1 (<i>m/z</i> 1081)												
Purple Ruffles	0.22	0.03	0.41	0.13	0.53	0.15	0.82	0.23	0.45	0.14	0.52 d	0.23
Rosie	0.38	0.06	0.47	0.11	0.44	0.06	0.73	0.25	0.31	0.09	0.49 d	0.20
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	0.18
Average	0.21 a	0.10	0.41 a	0.11	0.41 a	0.16	0.72 b	0.21	0.41 a	0.13		
anthocyanin 2 (<i>m/z</i> 1167)												
Purple Ruffles	0.08	0.03	0.25	0.08	0.31	0.08	0.44	0.11	0.16	0.04	0.27 d	0.14
Rosie	0.21	0.04	0.29	0.07	0.17	0.12	0.32	0.11	0.06	0.01	0.25 d	0.11
Sweet Petra Dark Red	0.05	0.01	0.20	0.07	0.26	0.03	0.28	0.06	0.12	0.02	0.15 e	0.07
Average	0.10 a	0.06	0.24 b	0.08	0.23 b	0.10	0.35 c	0.12	0.12 a	0.05		
anthocyanin 3 (<i>m/z</i> 1065)												
Purple Ruffles	0.89	0.07	1.61	0.33	2.02	0.55	1.80	0.57	1.18	0.47	1.58 d	0.57
Rosie	0.90	0.14	1.22	0.25	1.18	0.17	1.05	0.39	0.62	0.29	1.04 e	0.33
Sweet Petra Dark Red	0.41	0.02	1.26	0.33	1.03	0.49	1.14	0.29	0.83	0.20	0.87 e	0.28
average	0.66 a	0.24	1.34 b	0.33	1.38 b	0.61	1.31 bc	0.54	0.93 c	0.42		
anthocyanin 4 (<i>m/z</i> 1151)												
Purple Ruffles	0.37	0.04	0.98	0.25	1.18	0.31	0.92	0.25	0.35	0.13	0.81 d	0.40
Rosie	0.58	0.12	0.91	0.23	0.84	0.12	0.51	0.17	0.10	0.01	0.63 e	0.32
Sweet Petra Dark Red	0.21	0.01	0.78	0.22	0.59	0.32	0.51	0.20	0.21	0.05	0.41 f	0.19
average	0.33 a	0.14	0.86 b	0.24	0.85 bc	0.36	0.63 c	0.29	0.24 a	0.14		

^a Average concentrations with the same letter for a single anthocyanin are not statistically different.

^b n = 66 independent samples analyzed; 9 DAG, n = 9; 16, 23, and 35 DAG, n = 15; 49 DAG, n = 12.

Table 4

Average antioxidant capacities ($\bar{x} \pm$ standard deviation, SD) measured in trolox equivalents (TEAC, mmol/g dry weight, DW).^{a,b}

cultivar	days after germination											
	9		16		23		35		49		average	
	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Purple Ruffles	4.32	0.33	7.02	0.99	8.07	1.95	23.22	7.79	20.04	7.54	13.25	9.01
Rosie	5.14	0.85	7.38	0.92	7.57	0.73	28.73	12.80	27.72	3.76	15.09	12.21
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	9.51
average	4.32 a	0.86	8.39 b	2.98	7.60 b	1.29	26.06 c	8.36	23.27 c	6.23		

^a Average antioxidant capacities with the same letter for a single type of extract are not statistically different.

^b n = 66 independent samples analyzed; 9 DAG, n = 9; 16, 23, and 35 DAG, n = 15; 49 DAG, n = 12.