Anthocyanins in Basil (*Ocimum basilicum* L.)

Winthrop B. Phippen and James E. Simon*

Center for New Crops and Plant Products, 1165 Horticulture Building, Purdue University, West Lafayette, Indiana 47907-1165

The Lamiaceae family, which includes basil, sage, and thyme, has long been recognized as a rich source of diverse and unique anthocyanins. The development of intensely purple pigmented basil in the ornamental and herb trade prompted this examination of eight commercial varieties of purple basils (*Ocimum basilicum* L.) as a potential new source of anthocyanins. Anthocyanins were isolated and characterized utilizing high-performance liquid chromatography, spectral data, and plasma desorption mass spectrometry. Fourteen different anthocyanins were isolated, consisting of 11 cyanidin-based pigments and 3 peonidin-based pigments. The large-leaf basil varieties, Purple Ruffles, Rubin, and Dark Opal, had an average extractable total anthocyanin content ranging from 16.63 to 18.78 mg/100 g of fresh tissue, while the ornamental small-leaf variety, Purple Bush, had only 6.49 mg/100 g of fresh tissue. The highest concentration of total anthocyanins occurred just prior to flowering, although by day 8 seedlings already had accumulated all 14 anthocyanins. Comparisons were made to other anthocyanin sources, with results showing that purple basils are an abundant source of acylated and glycosylated anthocyanins and could provide a unique source of stable red pigments to the food industry.

Keywords: *Basil; Ocimum basilicum L.; anthocyanins; HPLC; plasma desorption mass spectrometry*

INTRODUCTION

The importance of anthocyanins as food colorings, UV protectants, inhibitors of pathogens, and medicinal compounds has been well documented with >300 anthocyanin compounds reported in plants (Harborne and Williams, 1995). Anthocyanins, which are responsible for the primary red and blue pigments in plants, have been recognized as contributing to plant growth, development, and reproduction (Holton and Cornish, 1995). These compounds have recently been shown to act as antioxidants, allowing for their potential use as a medicinal agent and as a food additive (Wagner, 1985; Tamura and Yamagami, 1994). Tsuda et al. (1994) found that anthocyanin pigments isolated from *Phaseolus vulgaris* L. showed significant antioxidative activity. Tsuda et al. (1996) were also able to demonstrate that anthocyanin pigments isolated from *P. vulgaris* L. had active oxygen radical scavenging activity and the ability to inhibit lipid peroxidases. The recent research on anthocyanins as antioxidant agents has increased the demand for unique and abundant sources of these compounds. Currently, *Perilla frutescens*, in the Lamiaceae family, is a known commercial source for anthocyanins. With the use of bioreactors and cell/tissue culture, 3 g of anthocyanins/L of medium was produced from *P. frutescens* var. Crispa (Zhong et al., 1991). The Lamiaceae family has many other plant species with high levels of anthocyanins (Saito and Harborne, 1992). Stable anthocyanins from *Ajuga* species (family Lamiaceae) were reported to be more stable in light when produced by in vitro extracts than in vivo extracts (Madhavi et al., 1996).

* Author to whom correspondence should be addressed [e-mail jesimon@hort.purdue.edu; fax (765) 494-0391].

The goal of our research was to investigate varieties of purple basil (*Ocimum basilicum* L.) as a potential new source of commercial anthocyanins. Characterization of anthocyanin pigments has not yet been reported in basil. Commercial purple basil varieties were also compared to known sources of anthocyanins to estimate potential yield and compositional differences among the different sources.

MATERIALS AND METHODS

Plant Tissue. Eight different varieties of purple basil (*O. basilicum*) were collected from various commercial seed sources in the United States. Some varieties were duplicated across seed sources due to phenotypic variations seen between the same variety from different sources. Plants were sown in the greenhouse and transplanted to the O'Neall Memorial Vegetable Research Farm (Lafayette, IN) on black plastic mulch in late June 1996. Plants were allowed to mature just prior to anthesis. Individual plant samples, each containing stems, flowers, and leaves, were collected from four plants of each variety. Harvested tissue samples were then placed in plastic bags, frozen in liquid nitrogen, and stored in a −20 °C freezer. Other anthocyanin sources [grape fruit (*Vitis labruscana* var. Concord), perilla leaves (*P. frutescens* var. Crispa), plum fruit (*Prunus domestica* L. var. Stanley), purple sage leaves (*Salvia officinalis* var. Purpurea), red cabbage leaves (*Brassica oleracea* var. Cardinal), and red raspberry fruit (*Rubus idaeus* var. Heritage)] were collected when the fruit and cabbage were ripe. Leaf tissue was harvested just prior to flowering. Three plants were sampled from each of the anthocyanin sources. All of the anthocyanin sources were collected from the Purdue Horticulture Research Farm and Gardens (West Lafayette, IN).

Anthocyanin Extraction. To prepare samples for HPLC analysis, the protocol established by Boss et al. (1996) was followed with some modifications. Frozen leaf tissue (2 g) was finely chopped and placed in a 15 mL centrifuge tube with 10

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Table 1. Comparison of Mean Total Extractable Anthocyanin Yields[a] among Commercial Purple Basil Varieties

variety	seed source	catalog no.	yield (mg/100 g of fresh tissue)
Dark Opal	Richters	S1260	16.32 ± 1.77
Dark Opal	Rupp Seeds	1094	18.73 ± 0.76
Holy Sacred Red	Rupp Seeds	1304	8.84 ± 0.59
Opal	Companion Plants	Oci 60x	12.77 ± 0.37
Opal	Nichols Garden Nursery	H003	11.71 ± 0.57
Osmin Purple	Johnny's	923	18.01 ± 0.80
Purple Bush	Richters	P1255-500	6.49 ± 1.10
Purple Ruffles	Richters	S1261	18.47 ± 0.71
Purple Ruffles	Shepherd's Garden Seeds	1368	17.91 ± 0.83
Purple Ruffles	Johnny's	905	15.87 ± 0.47
Purple Ruffles	Burpee	B-61341	18.78 ± 0.73
Red Rubin	Johnny's	924	17.44 ± 0.80
Red Rubin Purple Leaf	Shepherd's Garden Seeds	1365	18.33 ± 0.52
Rubin	Richters	S12260-500	17.38 ± 0.78
P. frutescens var. Crispa[b]	Shepherd's Garden Seeds	1793	18.08 ± 0.63

[a] Mean total extractable anthocyanin yield was determined by HPLC weight percents and reported as a mean of three individual plants ± SD. Known concentrations of cyanidin 3,5-diglucoside were used to calculate HPLC weight percents. [b] Used as a comparative standard.

mL of acidified methanol (0.1% HCl). The anthocyanins were extracted for 3 h at 4 °C. The leaf tissue was pelleted by centrifugation at 122000*g* for 10 min, and the supernatant was decanted to a 50 mL round-bottom flask. Samples were rotoevaporated to near dryness, resuspended in 1 mL of methanol, and stored at 4 °C until analyzed by HPLC. For preparative HPLC analysis, 40 g of tissue was extracted with 300 mL of acidified methanol (0.1% HCl) for 5 h at 4 °C. Samples were concentrated to 30 mL using rotary evaporation as described. For plums and grapes, only frozen fruit epidermal peels were utilized. For raspberries, the entire fruit was used. Whole leaf samples were used for perilla, red cabbage, and purple sage samples.

Mean total extractable anthocyanin yield was determined by HPLC weight percents and reported as a mean of three individual plants ± standard deviation (SD). Known concentrations of cyanidin 3,5-diglucoside were used to calculate HPLC weight percents.

Analytical HPLC Analysis. Analytical scale HPLC analysis was performed using a Varian (Varian Associates, Inc., Walnut Creek, CA) 9012 solvent delivery system and a Varian 9050 variable wavelength UV−vis detector connected to a Compaq 486 Prolinea computer with Varian Star version 4.5 integration computer software. A SynChropak (Micra Scientific, Inc., Northbrook, IL) RP-100 (C_{18}, 100 Å pores, 5 μm particles) column (250 × 4.6 mm) and guard column (50 × 4.6 mm) were used at 20 °C. Each run was monitored at 530 nm with a flow rate of 1.2 mL. A gradient elution was employed with initial conditions set at 30% solvent A (1.4% perchloric acid), 20% solvent B (100% methanol), and 50% solvent C (HPLC grade water). Solvent A was maintained at 30% throughout each elution. Solvent B was increased to 35% in 5 min and then increased to 55% in 35 min. After 35 min, solvent B was increased to 100% for 2 min and returned to initial conditions for 3 min, for a total run time of 40 min. In all cases, 20 μL of extract was injected onto the HPLC column. Perchloric acid was selected as the acidifying agent of the HPLC solvents because acetic acid was found to cause interference with plasma desorption (PD)-MS analysis (Wood et al., 1994).

Preparative HPLC Analysis. Preparative HPLC was run on a Waters (Waters Chromatography Division, Millipore Corp., Milford, MA) Delta Prep 4000 preparative chromatography system with a Waters 486 UV tunable detector connected to a Digital Venturis FX 5100 computer running Millennium version 2.75.01 integration software. A 30 mL aliquot of a concentrated crude plant extract was injected onto a Waters Prep NovaPak HRC_{18} 60 Å (6 μm, 40 × 100 mm) preparative cartridge column at 20 °C and 500 psi. The protocol for the analytical column separation was upgraded to a flow of 60 mL/min, and each anthocyanin peak was collected individually. Collected peaks were extracted on C_{18} solid-phase cartridges (900 mg capacity) (Baxter Healthcare Corp., Burdick and Jackson Division, Muskegon, MI) to remove the HPLC solvents. Anthocyanins were eluted from the cartridges with acidified methanol (0.1% HCl) and stored at 4 °C. Final concentrations of collected peaks were determined by utilizing a weight percent assay and the analytical HPLC protocol. Each individual peak was diluted to a concentration of 1 mg/mL and analyzed by PD-MS.

Spectral Data of Anthocyanin Extracts. Isolated peaks from the preparative HPLC were analyzed on a Shimadzu UV160U UV−vis recording spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, MD) from 200 to 600 nm. Samples were analyzed in quartz cuvettes in 0.1% HCl/MeOH. A wavelength maximum was determined for each isolated peak, along with known standards. Standards for cyanidin, cyanidin 3-glucoside, cyanidin 3,5-diglucoside, cyanidin 3-(*p*-coumarylglucoside), cyanidin 3-(*p*-coumarylglucoside)-5-glucoside, peonidin, peonidin 3,5-diglucoside, and peonidin 3-(*p*-coumarylglucoside)-5-glucoside were graciously donated by Dr. Geza Hrazdina, Cornell University. The elucidation of the aglycons, glucose residues, and acid residues on the cyanidin and peonidin structures was determined by acid and alkaline hydrolysis followed by HPLC analysis (Francis, 1982). For acid hydrolysis, an equal volume of 4 N HCl was added to each sample, which was then placed in a 100 °C water bath for 1 h. For alkaline hydrolysis, an equal volume of 2 N NaOH was added to each sample, which was placed at room temperature for 1 h. Samples were then analyzed by spectrophotometry and HPLC.

PD-MS of Anthocyanin Extracts. Plasma desorption mass spectra were obtained on a BioIon 20R plasma desorption mass spectrometer (Applied Biosystems, Foster, CA) utilizing a ^{252}Cf ion source and protocols established for anthocyanins (Wood et al., 1993). The resulting MeV fission fragments interact with the anthocyanin sample, producing ions that are mass analyzed by a time-of-flight mass spectrometer. Sample targets were prepared by electrospraying ≈50 μL of a 2 mg/mL nitrocellulose (Biotrace NT, Gellman Sciences, Inc., Ann Arbor, MI) solution in acetone onto a Mylar target (Applied Biosystems). Data were collected for 15 min at an accelerating voltage of 17 keV.

RESULTS AND DISCUSSION

Total Anthocyanins. Microscopic studies indicated that the color of purple basil plants results from the accumulation of anthocyanins in the epidermal layer of cells in stems, flowers, and, for the most part, leaves. Anthocyanin accumulation was also noted in the pith of the mature floral stems. All parts of the plant were used in the extraction protocol. Acidified methanol extractions from frozen and fresh tissue demonstrated no significant differences (data not shown). Total an-

Table 2. Characterization of Anthocyanins Extracted from Burpee's Purple Ruffles Basil[a] Based upon HPLC Retention Time (t_R), UV−Vis Spectral Data (λ_{max}), Molecular Weight ($[M]^+$), and Fresh Weight Percent (FW%)

peak no.	anthocyanin pigment	t_R (min)	λ_{max}[b] (nm)	$[M]^+$[c]	FW%[d]
1	cyanidin 3,5-diglucoside	8.8	285, 529	611	0.8
2	peonidin 3,5-diglucoside	10.8	282, 523	625	0.8
3	cyanidin 3-glucoside	13.3	289, 528	449	2.0
4	cyanidin based, *p*-coumaryl acid	16.0	283, 528	921	6.3
5	cyanidin based, *p*-coumaryl and malonyl acids	18.9	287, 528	1006	16.3
6	cyanidin (aglycon)	23.6	277, 539	287	0.6
7	cyanidin 3-(*p*-coumarylglucoside)-5-glucoside	24.6	281, 529	757	1.9
8	cyanidin based, *p*-coumaryl acid	25.2	292, 530	1084	11.0
9	cyanidin based, *p*-couomaryl and malonyl acids	28.0	292, 531	1169	5.3
10	peonidin (aglycon)	29.4	273, 524	301	0.4
11	cyanidin based, *p*-coumaryl acid	30.7	293, 531	1067	21.7
12	peonidin 3-(*p*-coumarylglucoside)-5-glucoside	31.5	284, 524	771	18.6
13	cyanidin based *p*-coumaryl and malonyl acids	33.1	294, 531	1152	18.6
14	cyanidin 3-(*p*-coumarylglucoside)	36.4	289, 529	595	0.8

[a] Anthocyanins were separated by gradient elution on a SynChropak C_{18} reversed phase column at a flow of 1.2 mL/min with detection at 530 nm. [b] Isolated peaks were analyzed in 0.1% HCl/MeOH and are listed in order of elution. [c] Calculated by PD-MS as $[M]^+$. [d] Calculated by weight percent from HPLC of 80-day-old tissue utilizing authentic standards.

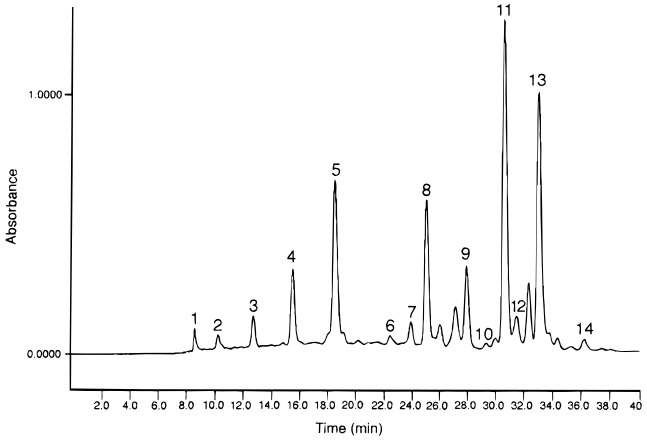


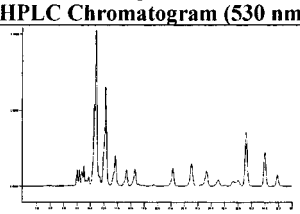
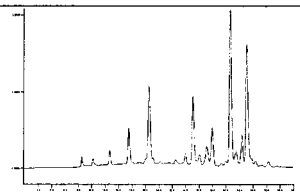
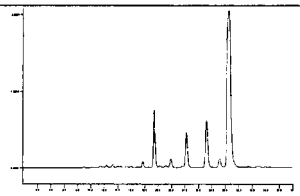
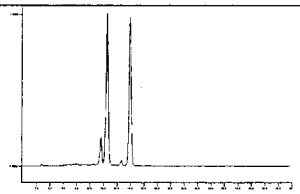
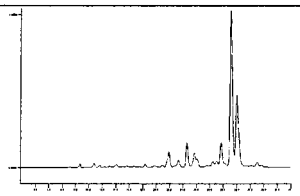
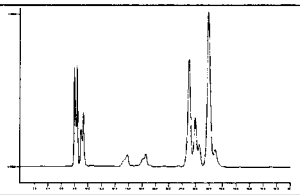
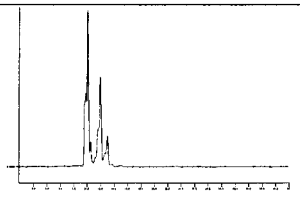
Figure 1. Representative HPLC chromatogram of anthocyanins isolated from Burpee's Purple Ruffles basil absorbing at 530 nm. Isolated peaks are numbered in the order of their elution and retention times.

thocyanin yield varied significantly between basil varieties ranging from 6.49 to 18.78 mg/100 g of fresh tissue (Table 1). Utilizing HPLC, observed peaks that absorb at 530 nm, a wavelength at which anthocyanins can be detected, were isolated from basil (Figure 1). Fourteen of the major peaks were characterized as anthocyanin pigments (Table 2). Following acid hydrolysis and when compared to known cyanidin and peonidin standards, the UV spectra and HPLC retention times of the isolated peaks indicated 11 of the identified peaks were cyanidin based, whereas the remaining 3 peaks were identified as being peonidin based. Eight of the HPLC peaks (peaks 1−3, 6, 7, 10, 12, and 14 in Figure 1 and Table 2) were identified by comparing retention times to known standards run under the same HPLC conditions. The identifications of individual peaks were later confirmed by isolating the individual peaks by preparative LC and comparing their UV−vis spectra to those of the known standards. The remaining six peaks (peaks 4, 5, 8, 9, 11, and 13) did not coincide with any available cyanidin- or peonidin-based standards. These six peaks were individually collected and analyzed by spectroscopy and PD-MS.

Malonic and *p*-coumaric acid were the only acids detected in the HPLC analysis of the alkaline hydrolysate. The major isolated pigment (t_R = 30.7 min) had UV_{max} at 293 and 330 nm and vis_{max} at 531. The ratio of E_{330}/E_{531} was 1.44, suggesting the presence of two acid residues according to Harborne (1958). The ratio of E_{440}/E_{531} was 10%, indicating glycosylation at the C3 and C5 positions. HPLC weight percents were determined by creating a standard curve for cyanidin 3,5-diglucoside of known concentrations.

Mass Spectrometry. A detection limit curve was established for the PD-MS protocol utilizing a cyanidin standard and the major pigment from basil (Table 2, peak 11). The pure standard of cyanidin had optimal detection at a concentration 0.5 mg/mL, while the less pure and more complicated major pigment from basil had an optimal detection at a concentration of 1.0 mg/mL. The higher concentration required for the basil anthocyanin is likely due to the impurities in the sample. The high concentrations of 0.5−1.0 mg/mL required for PD-MS prompted us to utilize preparative HPLC. The preparative HPLC technique was utilized solely to collect sufficient quantities of each pigment to measure on PD-MS. Yields and purities were not taken for each individual anthocyanin pigment. The $[M]^+$ values for all of the major peaks isolated from purple basil are provided in Table 2.

Anthocyanin Sources. Of the purple basils tested, differences were detected between the varieties for total anthocyanin content and anthocyanin profile (Table 1). Although total anthocyanin content varied among the varieties, each variety profile contained the same compounds but in slightly different proportions. The Purple Ruffles, Rubin, and Dark Opal varieties had consistently high levels of anthocyanins ranging from 16.63 to 18.78 mg/100 g of fresh tissue. The smaller bush type basil, Purple Bush, had the least amount of extractable anthocyanins at 6.49 mg/100 g of fresh tissue. On the basis of a weight percent HPLC assay (Figure 2), basil contained more total anthocyanins than perilla, plums, purple sage, red cabbage, and red raspberries but much less total anthocyanins than Concord grape skin peels. However, the total anthocyanin content reported here for grapes does reflect an inflated estimate, as only the skin peel was used. The HPLC chromatograms provided in Figure 2 illustrates the quantification and diversity of anthocyanin pigments seen across different natural plant sources. Simple mono- and diglycosidic pigments appear to have shorter retention times, such as found in raspberries and plums, whereas the more complex acylated and polyglycosylated pigments have longer retention times, as found in perilla and purple sage. When the types of accumulated anthocyanins are

Total Anthocyanin Concentration*	Profile of Anthocyanin Composition: HPLC Chromatogram (530 nm)
Grape *Vitis labruscana* 'Concord' 25.2 ± 2.89	
Purple Basil *Ocimum basicilcum* 'Purple Ruffles' 18.8 ± 0.73	
Perilla *Perilla frutescens* 'Crispa' 18.1 ± 0.63	
Plum *Prunus domestica* L. 'Stanley' 15.9 ± 2.12	
Purple Sage *Salvia officinalis* 'Purpurea' 7.8 ± 0.97	
Red Cabbage *Brassica oleracea* 'Cardinal' 7.8 ± 1.39	
Red Raspberry *Rubus idaeus* 'Heritage' 1.0 ± 0.67	

* Concentration reported as mg/100 g of fresh tissue.

Figure 2. Comparison of purple basil to other natural anthocyanin fruit and leaf sources for total anthocyanin yield and composition utilizing reversed phase HPLC at 530 nm. HPLC chromatograms indicate the diversity of anthocyanins found in each anthocyanin source. The total extractable yield is reported as a mean of three replications ± SD.

compared, basil varieties prove to be an abundant source of acylated and glycosylated anthocyanins. Cyanidin malonylated anthocyanins have been reported in cabbage, mustard, perilla, and onion (Mazza and Miniati, 1993). Individual pigments found in a wide variety of plant sources have been reviewed (Mazza and Miniati, 1993).

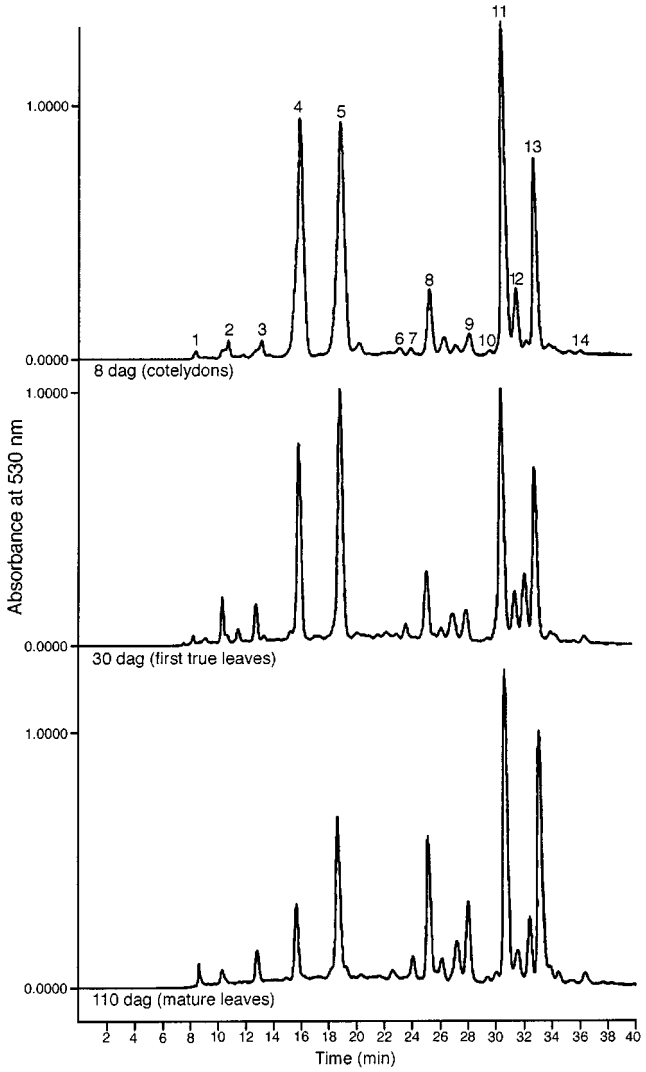


Figure 3. Accumulation of anthocyanins in field-grown basil from seedling to postflowering mature plants. Tissue was collected from the same plants at 8, 30, and 110 dag. Representative HPLC chromatograms of three replicates from each sampling time period are illustrated.

Accumulation of Anthocyanins in Basil. A time course experiment was conducted to investigate the synthesis of anthocyanins of field-grown purple basil throughout the growing season. Seedlings were sampled for total extractable anthocyanins at 8 (cotyledons), 30 (cotyledons and first true leaves), and 110 (mature leaves, harvest) days after germination (dag). When compared to 110 dag plant extracts (Figure 3), 8-day-old seedlings contained all 14 isolated anthocyanins. However, only 4 (peaks 4, 5, 11, and 13) of the 14 isolated anthocyanins are present in abundance in the 8-day-old seedlings. As plants mature, the less acylated forms of anthocyanins, cyanidin 3-glucoside and cyanidin 3,5-diglucoside (peaks 3 and 1), begin to appear. Perhaps these are the initial steps in anthocyanin degradation during plant maturity. The determination of which anthocyanin type was present during the growth cycle of a plant will enable the extraction of highly acylated and glycosylated anthocyanins for increased stability. Anthocyanins with glucose residues have been shown to have increased stability to temperature and light when they are acylated (Brouillard, 1982).

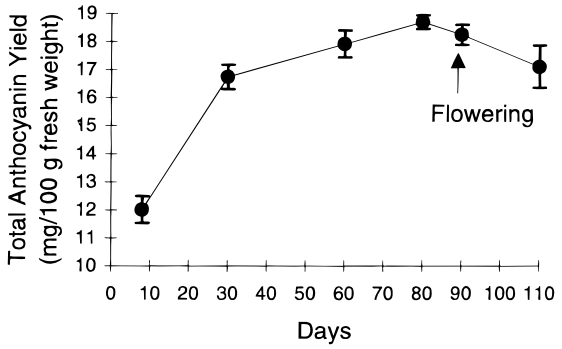


Figure 4. Accumulation of total anthocyanins from Burpee's Purple Ruffles basil over a single growing season, in central Indiana, 1996. Data points represent the average of three replicates, and error bars indicate standard deviation.

There was no other significant change in the HPLC profile between 30 and 110 dag. The total extractable anthocyanin yield did vary across the sampling times throughout the growing season (Figure 4). At 8, 80, and 110 dag, 12.01 ± 0.48, 18.70 ± 0.25, and 17.11 ± 0.76 mg/100 g of fresh weight, respectively, the highest concentration of anthocyanins occurred just prior to flowering at 90 dag. There was a slight decrease in anthocyanin content once flowering occurred through the final harvest at 110 dag.

Stable, available, and inexpensive sources of anthocyanins are attractive for use in the food industry and for medicinal purposes. Our results show that purple basil varieties can serve as a significant source of total anthocyanins. Though the skin peels of grapes contained more total anthocyanins, the relatively inexpensive commercial production of basil suggests that purple basil should be considered as a cost-effective source of anthocyanins. The abundance of acylated and glycosylated anthocyanins in basil extracts may also offer a unique source of stable red pigments for the food industry. Identification of high-yielding sources of anthocyanins could enable researchers to continue work on the medicinal and antioxidant properties of these compounds. Research in our laboratory is currently focusing on the production of anthocyanins in culture, for comparisons of in vivo and in vitro stability of anthocyanin production from basil.

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