

Analysis and Identification of Anthocyanins in Petunia Petals

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ABSTRACT

Anthocyanins are a group of water-soluble pigments found in fruits, vegetables, and flowers that give these plants their brilliant colors. In addition to their colorful characteristics, anthocyanins possess antioxidant properties that have been implicated in disease prevention. The analysis and identification of the anthocyanins present in Petunia Hybrida Blue Wave and Petunia Surfinia Sky Blue was conducted utilizing liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS). The anthocyanins were extracted from the petunia petals and separated by reverse-phase chromatography prior to being introduced into the mass spectrometer. The characteristic purple color of the Blue Wave is due to a mixture of cyanidin and malvidin containing anthocyanins, whereas the light blue color of the Sky Blue variety is due to the presence of primarily delphinidin containing anthocyanins. The core structures are highly glycosylated and these sugars are acylated to yield a complex mixture of pigment molecules.

INTRODUCTION

Petunia Hybrida Blue Wave and Petunia Surfinia Sky Blue are available through most retailers. The characteristically deep purple color of Blue Wave and the light blue color of Sky Blue are due to anthocyanins, a large family of glycosylated polyhydroxy and polymethoxy derivatives of flavylum salts. Anthocyanins, in fact, are responsible for many of the fruit and floral colors in nature. Initially, anthocyanins were characterized through the use of paper chromatography. This gave way to the utilization of reversed-phase HPLC coupled with UV-Vis detection. However, discrepancies between many studies using this method exist because comparing retention times and UV-vis spectra alone is frequently insufficient for differentiating pigments with similar structures. In an effort to remedy its shortcomings, researchers have combined this methodology with electrospray ionization mass spectrometry (ESI-MS). ESI, a highly sensitive, mild, ionization technique has since proven to be a very powerful tool for anthocyanin characterization. Extensive work has been conducted to elucidate not only what anthocyanins are present in nature but also, which anthocyanins are characteristic of various flora. The purpose of this study is the characterization and identification of anthocyanins present in Blue Wave and Sky Blue petunias.

EXTRACTION AND ISOLATION OF ANTHOCYANINS.

Fresh petunia petals were treated with 50/50 methanol/ water with 0.1% formic acid, ground with a glass stirring rod and placed in sonicator for one hour to extract the anthocyanins. The mixture was placed in a glass-sintered funnel and the filtrate was washed three times with an equal volume of 50/50 ethyl acetate/cyclohexane, to remove flavinoid compounds. A small aliquot of the aqueous fraction was filtered through a 0.2 μm nylon syringe filter (Whatman Inc., Clifton, NJ) prior to introduction into the LC.

INSTRUMENTATION – HPLC/DAD/ESI-MS/MS ANALYSES

LC/ESI-MS/MS experiments were performed on an Agilent MSD XCT ion trap mass spectrometer (Palo Alto, CA) equipped with an electrospray ionization (ESI) interface, 1100 HPLC, a DAD detector, and Chemstation software. The column used was a 150 x .5 mm i.d., Zorbax SB- C18 5 μm (Agilent, Palo Alto, CA). Solvents were (A) 0.1% formic acid/ 99.9% water (v/v) and (B) 0.1% formic acid/ 99.9% acetonitrile (v/v). Solvent gradient was 0-20 min, 10-50% B; 20-31 min, 50-10% B; and 31-35 min, 10% B. Flow rate was 6.000 $\mu\text{L}/\text{min}$, injection volume was 0.5 μL , and column temperature was 25 $^{\circ}\text{C}$. The ESI parameters were as follows: nebulizer, 13 psi; dry gas (N₂), 4.00 L/min; dry temperature, 325 $^{\circ}\text{C}$; trap drive, 76.5; skim 1, 40 V; lens 1, -5.00 V; octopole RF amplitude, 150 Vpp; capillary exit, 158.5 V. The ion trap mass spectrometer was operated in positive ion mode scanning from m/z 100 to m/z 2200 at a scan resolution of 13000 amu/s. Trap ICC was 30000 units and maximal accumulation time was 300000 μs . MS-MS was operated at a fragmentation amplitude of 1.2 V, and threshold ABS was 3,000,000 units.

FIGURE 1. MS/MS CHROMATOGRAPH BLUE WAVE

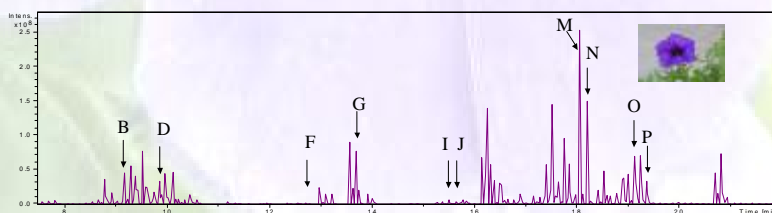
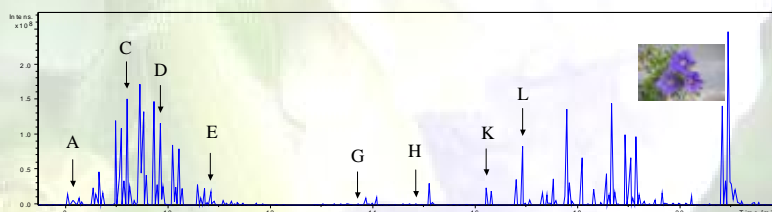
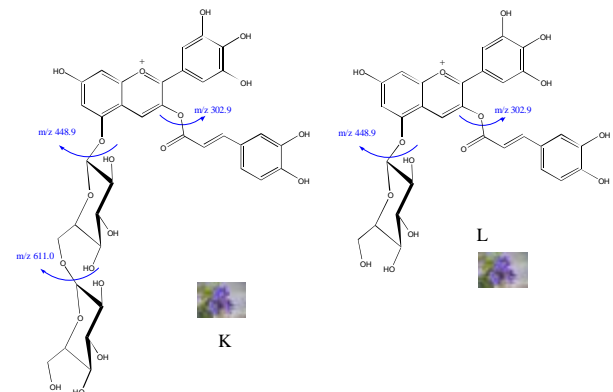
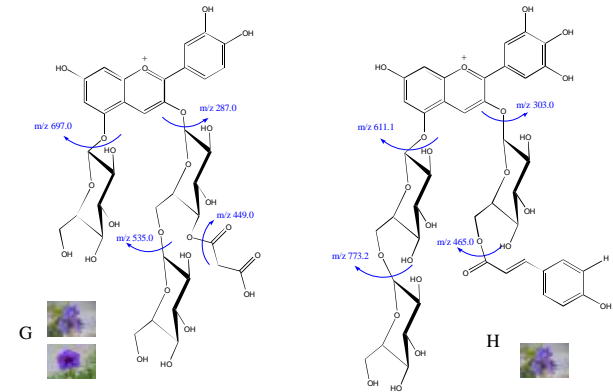
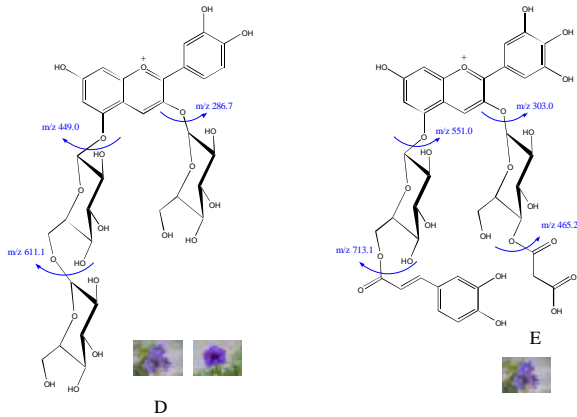
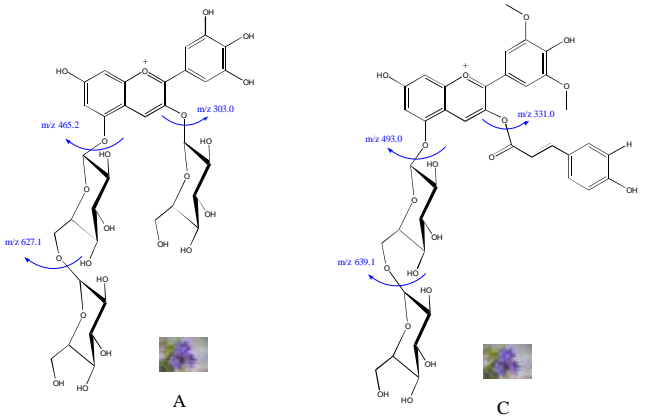
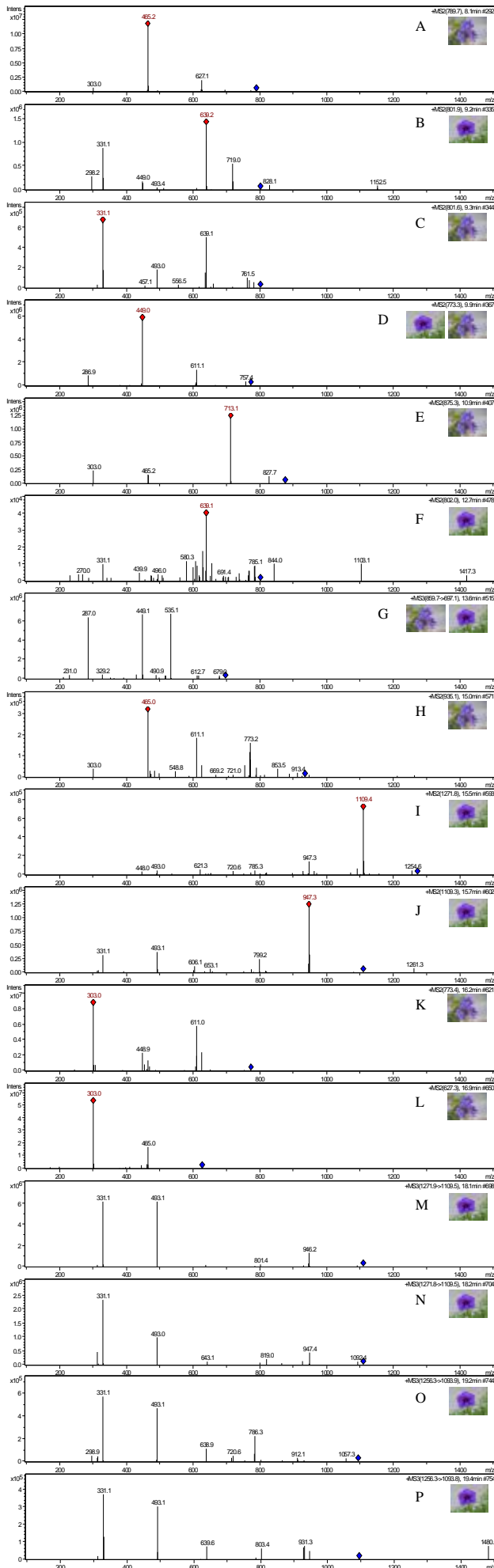


FIGURE 2. MS/MS CHROMATOGRAPH SKY BLUE





RESULTS

Anthocyanin pigments in Petunia Hybrida Blue Wave and Petunia Surfinia Sky Blue were separated by RP-HPLC and the identity of the compounds were elucidated by MS and MS/MS spectra. Sixteen pigments were identified in the two petunias and corresponded to peaks A-P. Two of the sixteen pigments were found in both varieties (D & G). The remaining peaks in the two LC separations (Figures 1 & 2) exhibited molecular ions and subsequent fragmentation patterns inconsistent with those of previously reported pigments. Identification of the peaks was achieved with the aid of existing literature mass spectral analysis. The molecular ions and product ions for the sixteen peaks are summarized in Table 1.

TABLE 1. PROPOSED STRUCTURES FOR ANTHOCYANIN PIGMENTS

		COMPOUND	RT	M+	PRODUCT IONS
SB	A	Delphinidin-3-glucose-5-diglucoside	8.1	790	303,465,627
BW	B	Malvidin-3-coumaryl-5-diglucoside	9.2	802	331,493,639
SB	C	Malvidin-3-coumaryl-5-diglucoside	9.3	802	331,493,639
SB BW	D	Cyanidin-3-diglucoside-5-glucose	9.9	773	287,449,611
SB	E	Delphinidin-3-malonylglucose-5-caffeoylglucose	10.9	875	303,465,551,713
BW	F	Malvidin triglucoside	12.7	802	331,496,639,782
SB BW	G	Cyanidin-3-malonyldiglucoside-5-glucose	13.5	860	287,449,535,697
SB	H	Delphinidin-3-caffeoylglucose-5-diglucoside	15.0	935	303,465,611,773
BW	I	Malvidin-3-caffeoyldiglucoside-5-caffeoyldiglucoside	15.5	1271	331,493,785,947,1109,
BW	J	Malvidin-3-caffeoyldiglucoside-5-caffeoylglucose	15.7	1109	331,493,799,947
SB	K	Delphinidin-3-caffeoyl-5-diglucoside	16.2	774	303,449,611
SB	L	Delphinidin-3-caffeoyl-5-glucose	16.9	627	303,465
BW	M	Malvidin-3-caffeoylrhamnosylglucose-5-caffeoylglucosylrhamnose	18.1	1271	331,493,639,801,946,1109
BW	N	Malvidin-3-catechin-5-feruloyldiglucoside	18.2	1271	331,493,643,819,1109
BW	O	Malvidin-3-caffeoylglucose-5-catechinyldiglucoside	19.2	1256	331,493,639,786,912,1093
BW	P	Malvidin-3-caffeoylglucose-5-catechinyldiglucoside	19.4	1256	331,493,639,803,931,1093

DISCUSSION

We have identified and proposed structures for the anthocyanins from Blue Wave and Sky Blue Petunias. These structures are consistent with the observed fragmentation patterns and with the order of chromatographic separation. The core molecule in each case is easily identified as either cyanidin, malvidin, or delphinidin. However, it should be noted that the structures could differ from the predictions with respect to points of attachment to the carbohydrate molecules.

The peaks D & G were found in both Blue Wave and Sky Blue petunias and were both cyanidin based compounds. Cyanidin is typically associated with red-colored flowers and may be present as an artifact of petunia crosses. The remainder of the compounds were divided into delphinidin-based anthocyanins in Sky Blue and malvidin-based anthocyanins in Blue Wave. This observation is well-supported by existing evidence with respect to blue flowers being high in delphinidin-based anthocyanins and purple flowers being rich in malvidin-based anthocyanins.

FUTURE WORK

We are going to continue with our work on the identification of anthocyanins from Petunia and other flowers of interest. We plan to scale up our work and use preparative RP-HPLC to isolate the anthocyanins in sufficient quantities to allow for NMR spectral analysis.