

## Characterization Of Anthocyanins In *Petunia Hybrida* 'Purple Wave'

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### Abstract

Four water-soluble pigments extracted from *Petunia hybrida* 'Purple Wave', a variety of purple petunia, were characterized. The pigments belong to the anthocyanins, which are a group of naturally occurring phenolic compounds responsible for the color of many plants, flowers, and fruits. The structures of the pigments were established by UV-Visible and LC-MS techniques. The pigments identified are all derivatives of malvidin exhibiting cis-trans isomerism about a side-chain double bond as well as varying numbers of conjugated carbohydrate substituents.

### Introduction

*Petunia hybrida* Purple Wave is a hybrid species available through most retailers. The characteristically deep purple color of the flower is due to the presence of 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.<sup>1</sup>

Initially, anthocyanins were characterized through the use of paper chromatography.<sup>2</sup> This gave way to the utilization of reversed-phase HPLC coupled with UV-Vis detection.<sup>3-5</sup> However, discrepancies between many studies using this method exist because comparing retention times and UV-Vis spectra alone are 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.<sup>6-9</sup>

Extensive work has been conducted to elucidate not only what anthocyanins are present in nature but also, which anthocyanins are characteristic of various flora.<sup>10-13</sup> The purpose of this study is the characterization and identification of anthocyanins present in Purple Wave.

### Materials and Methods

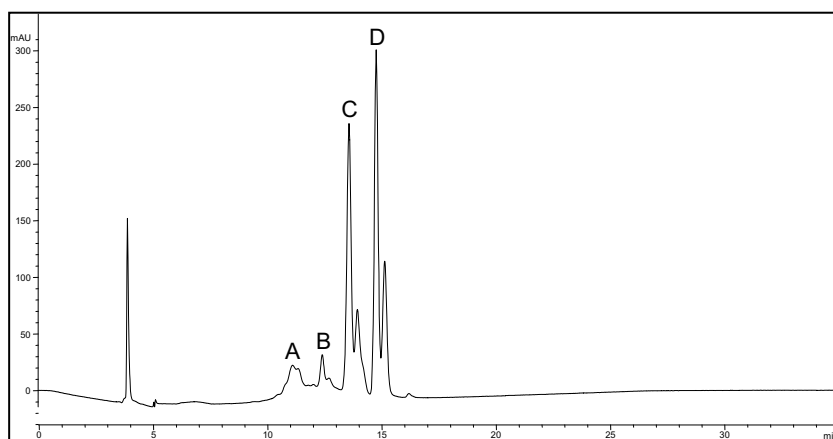
**Extraction and Isolation of Anthocyanins.** Fresh petunia petals were treated with 50/50 methanol/water with 0.1% formic acid, ground with a glass stirring rod and placed in a sonicator for one hour to extract the anthocyanins. The mixture was placed in a separatory funnel and the filtrate was washed 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  $\mu\text{m}$  nylon syringe filter (Whatman Inc., Clifton, NJ) prior to introduction into the LC.

HPLC/DAD/ESI-MS/MS Analyses. LC/ESIMS/MS experiments were performed on an Agilent MSD SE 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 0.5 mm i.d., Zorbax SB-C18 5  $\mu$ m (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  $\mu$ L/min, injection volume was 0.5 $\mu$ L, and column temperature was 25°C. The ESI parameters were as follows: nebulizer, 13 psi; dry gas (N<sub>2</sub>), 4.00 L/min; dry temperature, 325°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 30,000 units and maximal accumulation time was 300000  $\mu$ s. MS-MS was operated at a fragmentation amplitude of 1.2 V, and threshold ABS was 3,000,000 units.

Solvents. Ethyl acetate, formic acid, and cyclohexane were reagent grade (Sigma Aldrich). Water and acetonitrile (Burdick and James) were HPLC grade.

## Results and Discussions

Anthocyanin pigments in *Petunia Hybrida* Purple Wave were separated by RP-HPLC and the identities of the compounds were elucidated by MS and MS/MS spectra. Four pigments were identified and corresponded to peaks A-D in Figure 1.



**Figure 1.** RP-HPLC trace of Purple wave extract measured at 530nm.

The remaining peaks exhibited molecular ions and subsequent fragmentation patterns inconsistent with those of previously reported pigments. Identification of the peaks (A-D) was achieved with the aid of existing literature mass spectral analysis.<sup>12</sup> The molecular ions and product ions for the four peaks are summarized in TABLE 1.

A	1125.0	963.0	801.0	639.0	492.9	330.9
B	1109.0	947.0	785.0	639.0	492.9	331.0
C		963.0	801.2	639.1	493.1	330.9
D		947.0	785.2	639.1	493.1	330.9

**TABLE 1.** Summary of peaks A-D with corresponding molecular and product ions.

The MS spectrum of peak A yielded a molecular ion of  $m/z$  1125.0 and the MS/MS spectrum of peak A yielded product ions of  $m/z$  963.0 ( $[M-C_6H_{10}O_5]^+$ ), 801.0( $[M-C_6H_{10}O_5-C_6H_{10}O_5]^+$ ), 639.0( $[M-C_6H_{10}O_5-C_6H_{10}O_5-C_9H_6O_3]^+$ ), 492.9( $[M-C_6H_{10}O_5-C_6H_{10}O_5-C_9H_6O_3-C_6H_9O_4]^+$ ), and 330.9( $[M-C_6H_{10}O_5-C_6H_{10}O_5-C_9H_6O_3-C_6H_9O_4-C_6H_{10}O_5]^+$ ). The ions corresponded to malvidin 3-O-(6-O-(4-O-(4-O-glucopyranosyl)caffeoyl)rhamnosyl)glucopyranoside]-5-O-glucopyranoside, malvidin 6-O-(4-O-(4-O-glucopyranosyl-caffeoyl)rhamnosyl)-3-O-glucopyranoside, malvidin 6-O-(4-O-caffeoyl-rhamnosyl)-3-O-glucopyranoside, malvidin (6-O-rhamnosyl)-3-O-glucopyranoside, malvidin 3-O-glucopyranoside, and malvidin, respectively. Refer to Figure 2.

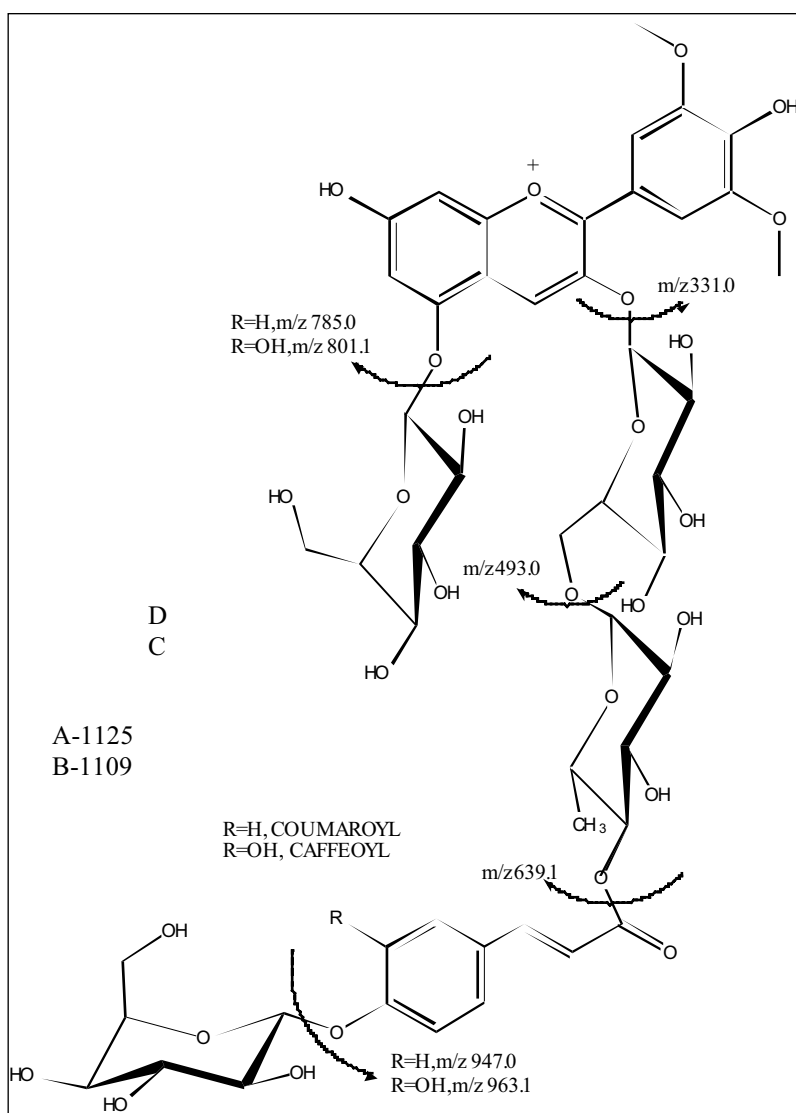
The MS spectrum of peak B yielded a molecular ion of  $m/z$  1109.0 and the MS/MS spectrum of peak B yielded product ions of  $m/z$  947.0( $[M-C_6H_{10}O_5]^+$ ), 785.0( $[M-C_6H_{10}O_5-C_6H_{10}O_5]^+$ ), 639.0( $[M-C_6H_{10}O_5-C_6H_{10}O_5-C_9H_6O_2]^+$ ), 492.9( $[M-C_6H_{10}O_5-C_6H_{10}O_5-C_9H_6O_2-C_6H_9O_4]^+$ ), and 331.0( $[M-C_6H_{10}O_5-C_6H_{10}O_5-C_9H_6O_2-C_6H_9O_4-C_6H_{10}O_5]^+$ ). The ions corresponded to malvidin 3-O-(6-O-(4-O-(4-O-glucopyranosyl) coumaroyl) rhamnosyl)glucopyranoside]-5-O-glucopyranoside, malvidin [6-O-(4-O-(4-O-glucopyranosyl) coumaroyl) rhamnosyl]-3-O-glucopyranoside, malvidin [6-O-(4-O-coumaroyl-rhamnosyl)] -3-O-glucopyranoside, malvidin 3-O-(6-O-rhamnosyl) gluco- pyranoside, malvidin 3-O-glucopyranoside, and malvidin, respectively. The difference in molecular weights of the molecular ions of pigment A and pigment B can be accounted for the differing structures of the acyl group at position four of the rhamnose substituent. This was confirmed by the converging molecular weights of the two product ions of pigments A and B after the removal of this acyl group. The difference is a molecular weight of sixteen. This is expected, whereas the caffeoyl moiety contains additional hydroxyl substitution on carbon three of the benzene ring. The additional hydroxyl group of pigment A imparts a higher polarity than pigment B, which is consistent with the chromatography.

The MS spectrum of peak C yielded a molecular ion of  $m/z$  963.0 and the MS/MS spectrum of peak C yielded product ions of  $m/z$  801.2( $[M-C_6H_{10}O_5]^+$ ), 639.1( $[M-C_6H_{10}O_5-C_9H_6O_3]^+$ ), 493.1( $[M-C_6H_{10}O_5-C_9H_6O_3-C_6H_9O_4]^+$ ), and 330.9( $[M-C_6H_{10}O_5-C_9H_6O_3-C_6H_9O_4-C_6H_{10}O_5]^+$ ). This peak exhibited the same fragmentation pattern as peak A with the exception of  $m/z$  of the molecular ion being 163 less. This weight corresponds to glucose and therefore allows us to report that pigment C has the same structure as pigment A without the additional glycosylation at carbon four of the caffeoyl moiety. The lower polarity of the proposed molecule is consistent with its delayed elution.

The MS spectrum of peak D yielded a molecular ion of  $m/z$  947.0 and the MS/MS spectrum of peak D yielded product ions of  $m/z$  785.2( $[M-C_6H_{10}O_5]^+$ ), 639.1( $[M-C_6H_{10}O_5-C_9H_6O_2]^+$ ), 493.1( $[M-C_6H_{10}O_5-C_9H_6O_2-C_6H_9O_4]^+$ ), and 330.9( $[M-C_6H_{10}O_5-C_9H_6O_2-C_6H_9O_4-C_6H_{10}O_5]^+$ ).

This peak exhibited the same fragmentation pattern as peak A with the exception of  $m/z$  of the molecular ion being 163 less. This weight corresponds to glucose and therefore allows us to report that pigment D has the same structure as pigment B without the additional glycosylation at carbon four of the coumaroyl moiety. The lower polarity of the proposed molecule is consistent with the chromatography.

**Figure 2.** Pigment molecules and mass spectral fragmentation.



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