

Identification of Anthocyanins? in Cactaceae by LC-ESI-MS-MS

Charles A. Johnson, Paul A. Campbell, Mindy Walker, Chad M. Scholes, and James M. Chapman
Rockhurst University, Kansas City, MO



ABSTRACT

Anthocyanins and betalains are water soluble vacuolar pigments. In flowers, anthocyanin and betalain pigments function as pollinator attractants, and in fruits, the colorful skins attract animals which will eat the fruits and disperse the seeds. In photosynthetic tissues (such as plant leaves or the stems of cacti), anthocyanins and betalains have been shown to act as a "sunscreen", protecting cells from photodamage by absorbing UV and blue-green light, thereby protecting the tissues from photoinhibition, or high light stress. They are synthesized exclusively by organisms of the plant kingdom, and have been observed to occur in all tissues of higher plants, providing color in leaves, stems, roots, flowers, and fruits. While the majority of land plants contain anthocyanins, there are a few examples of plants producing betacyanins as in the *Caryophyllales*, *Cactus* and *Galium* families. This work began as the characterization of betalain pigments from Beehive cactus (*Mammillaria vivipara* var. *vivipara*) flower petals by LC-ESI-MS-MS. In addition to the expected betalains several anthocyanins were unexpectedly identified in the flower petals. The identification of these anthocyanins in *Mammillaria Vivipara* var. *vivipara* is a novel discovery in the species. Additional work has since been carried out on 20 different cacti flower petals encompassing five genera of cacti, all of which have been found to contain anthocyanins. Approximately 40 different anthocyanins have been identified in the extracts of cacti flower petals at this stage of the work. Comparisons to anthocyanin standards obtained, from plants known to contain anthocyanins, has resulted in the identification of 15 of these pigments to this point.

INTRODUCTION

These pigments are synthesized by the plant kingdom, which have been observed in the tissues of higher plants. They are responsible for the color in leaves, stems, roots, and flowers and fruits. The chemical structure of anthocyanidins are based upon the flavonoid family of molecules that is in turn based on the C6-C3-C6 configuration in the flavan nucleus. Figure 1 shows the structure of the anthocyanidins identified. Anthocyanins are anthocyanidins linked with one or more sugar moieties. The most common sugars are glucose, galactose, rhamnose, and arabinose. These sugars can also be acylated by acetic acid, malonic acid, and coumaric acid. Betacyanins have been identified in *Caryophyllales*, *Cactus* and *Galium mollugo* which are based upon betalamic acid (FIGURE 2) and vary depending on the components bonded to the main structure. Betacyanins (FIGURE 3) are formed when the group is 3,4-dihydroxyphenylalanine, which may or may not be glycosylated.

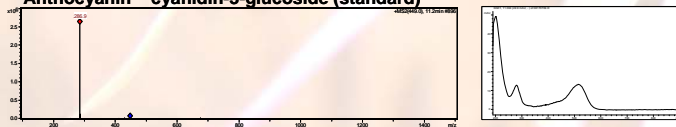
EXTRACTION AND ISOLATION OF PIGMENTS

Fresh flowers and fruits from several varieties of *Mammillaria* and *Opuntia* genera were extracted by means of a procedure which treated the flowers and fruits 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 betacyanins, anthocyanins, and flavonols. 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 µL/min, injection volume was 0.5 µL, and column temperature was 25 °C. The ESI parameters were as follows: nebulizer, 13 psi; dry gas (N₂), 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 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.

Anthocyanin – cyanidin-3-glucoside (standard)



MASS SPECTRAL AND UV DATA

No.	RT	Structure	UV	LC/MS (M+H) M/Z	MS/MS M/Z	Source
1	15.0	Luteolin/kampferol-glucose-glucose	A	611	287, 449, 611	M. pringlei v. longispina
2	15.4	Isorhamnetin-glucose-rhamnose-glucose	A	787	317, 479, 625, 787	M. columbiana yucatanensis
3	16.1	Quercetin-rhamnose-glucose-glucose	A	773	303, 449, 611, 773	Unknown Mammillaria
4	16.1	Luteolin/kampferol-glucose-xylose-glucose	A	697	287, 449, 535, 697	M. pringlei v. longispina
5	16.2	Quercetin-glucose-rhamnose-glucose	A	773	303, 465, 611, 773	Echinocereus subinermis v. aculeatus
6	16.2	Luteolin/kampferol-xylose-rhamnose-glucose	A	727	287, 419, 727	Mammillaria flavicentra
7	16.7	Quercetin-glucose-rhamnose-xylose	A	744	303, 465, 611, 744	Mammillaria theresae
8	16.8	Quercetin-glucose-rhamnose-xylose	A	744	303, 465, 611, 744	Mammillaria theresae
9	17.0	Quercetin-glucose-rhamnose-xylose	A	744	303, 465, 611, 744	Mammillaria theresae
10	17.1	Quercetin-glucose-rhamnose	A	611	303, 465, 611	Mammillaria rhodantha
12	17.1	Quercetin-glucose-rhamnose	A	611	303, 465, 611	Mammillaria spinosissima
12	17.3	Luteolin/kampferol-glucose-rhamnose-glucose	A	757	287, 449, 595, 757	Mammillaria theresae
13	17.7	Luteolin/kampferol-glucose-glucose-glucose	A	773	287, 611, 773	Unknown Mammillaria
14	17.7	Luteolin/kampferol-glucose-rhamnose-rhamnose	A	741	287, 449, 595, 741	M. columbiana yucatanensis
15	17.8	Luteolin/kampferol-glucose-rhamnose-xylose	A	727	287, 449, 595, 727	Mammillaria theresae
16	18.0	Luteolin/kampferol-glucose-rhamnose-gallic acid	A	741	287, 449, 595, 741	M. pringlei v. longispina
17	18.3	Luteolin/kampferol-xylose-xylose	A	551	287, 419, 551	Mammillaria occidentalis
18	18.5	Quercetin-glucose	A	465	303, 465	Mammillaria spinosissima
19	18.6	Quercetin-glucose	A	465	303, 465	Sulcorebutia menepesii
20	18.6	Quercetin-glucose-rhamnose	A	611	303, 464, 611	Mammillaria vivipara
21	18.6	Quercetin-glucose-rhamnose	A	611	303, 464, 611	Mammillaria flavicentra

FIGURE 1.
Anthocyanidin

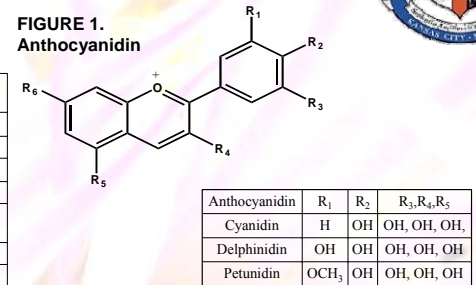


FIGURE 2.
Betalamic acid

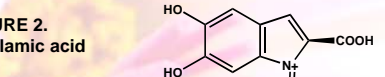
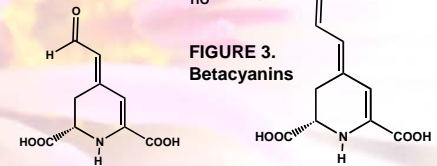
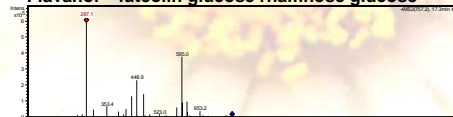


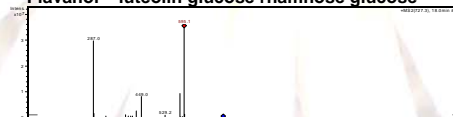
FIGURE 3.
Betacyanins



Flavanol – luteolin glucose rhamnose glucose



Flavanol – luteolin glucose rhamnose glucose



Flavanol– quercetin rhamnose glucose



Betacyanin – betanin



CONCLUSIONS

Commercially purchased flavonoid and anthocyanin standards exhibit very discernible differences in their UV/Visible spectra. Characteristically, the flavonols have absorbance maxima of 240-270 nm and 340-370 nm and the anthocyanins exhibit maxima of 280-300 nm and 500-540 nm. The betacyanins exhibit maxima of 260-270 and 510-530 nm. The retention times on RPLC of the three different classes overlap considerably. There are variations dependent upon substitution and acylation, but in general the betacyanins are found between 5-20 minutes, the anthocyanins between 6-20 minutes, and the flavonols between 15-25 minutes. The components eluting between 6-20 minutes with the secondary absorbance near 500-530 nm can be attributed to the presence of betacyanins or anthocyanins. The components can be differentiated by the mass spectral profile. Our initial work seemed to indicate the presence of anthocyanins in the flowers and fruits of the cactus. However, we have since discovered that the co-eluting betacyanin and flavanol glycoside components lead us to erroneously believe that there were anthocyanins in the cacti. We have not been able to eliminate all of the tentative anthocyanins at this point, but we have reduced the suspected number. This recent work has allowed us to identify several novel betacyanins and flavanol glycosides.

FUTURE WORK

We have recently developed a procedure for the rapid separation of the betacyanins and anthocyanins from the flavonols utilizing DSC-MCAX SPE cartridges from Supelco and plan to isolate the two components prior to analysis by LC-MS. This should significantly reduce the number of co-eluting peaks and make the identification of the betacyanins and flavanol glycosides easier.

REFERENCES

- Dieter Strack, Thomas Vogt, and Willibald Schliemann. Recent advances in betalain research. *Phytochemistry* 62 (2003) 247-269.
- Miller, J. M. 1988. Floral pigments and phylogeny in *Echinocereus* (Cactaceae). *Systematic Botany* 13(2): 173-183.
- Naoko Kobayashi, Jurgen Schmidt, Manfred Nimtz, Victor Wray, and Willibald Schliemann. Betalains from Christmas cactus. *Phytochemistry* 54 (2000) 419-426