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IDENTIFICATION AND QUANTIFICATION OF ANTHOCYANINS IN MUSCADINE GRAPES BY HPLC AND HPLC-MS

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ABSTRACT

Total anthocyanin content and individual anthocyanin profile of ten cultivars of muscadine grapes were assessed. Total anthocyanin content was determined by a pH differential method. Individual anthocyanins were analyzed by HPLC and their identity confirmed by HPLC-MS. The total anthocyanin content and the sum of the individual anthocyanins had a high correlation (R = 0.98). The average anthocyanin content of muscadine grapes was lower than published values for red European and other American red grapes. However, the purple muscadine grapes have anthocyanins levels that may be considered important from the nutraceutical point of view.

Keywords: anthocyanins, food analysis, muscadine grapes, polyphenols, *vitisrotundifolia.*

1. INTRODUCTION

Muscadine grapes *(Vitisrotundifolia*Michx.) are indigenous to the southeastern United States. Muscadines are vigorous vines that may grow up to 30 meters in the wild. They differ botanically from other grapes and are placed in a separate sub-genus, *Muscadinia.* Muscadine fruits are round, 2.5 to 3.5 cm in diameter with thick, tough skin and may have up to 5 seeds (Pastrana-Bonilla, Akoh, Sellappan, and Krewer, 2003).

Anthocyanins are water soluble, glycosy1ated derivatives based on the cyanidinaglycon(Chandra, Rana, and Li, 2001; J.P. Goiffon, M. Brun, and M.J. Bourrier, 1991) and are part of the flavonoid family. These pigments are responsible for the red, purple and blue colors of most fruits and flowers (da Costa, Nelson, Margolis, and Derek, 1998). Anthocyanins have the potential to be used as natural food colorants (Wu *et al.*, 2014). However, enzymes, pH, temperature, and oxygen affect their color quality (Chandra *et al.*, 2001; Wang, Tong, Chen, and Gangemi, 2010). Co-pigmentation with other phenolic compounds may increase their stability (Darias-Martín, Carrillo, Díaz, and Boulton, 2001). Anthocyanins are part of the human diet and they occur in many fruits and vegetables (Sellappan, Akoh, and Krewer, 2002). The average daily intake of anthocyanins in the United States was estimated at 215 mg during the summer and 180 mg during the winter (Clifford, 2000). The beneficial antioxidant activity and therefore, positive health effects of anthocyanins are a significant added value for their use as food colorants (Wu *et al.*, 2014). The antioxidant function of anthocyanins seems to be related to their hydrogen donation capacity, metal chelation and protein binding (SatueGracia, Heinonen, and Frankel, 1997). Anthocyanins have been found to be powerful antioxidants in comparison to other common antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and a-tocopherol (Espín, Soler-Rivas, Wichers, and García-Viguera, 2000; Narayan, Akhilender Naidu, Ravishankar, Srinivas, and Venkataraman, 1999). Anthocyanin-rich fruit extracts have been used in traditional medicine as anti-inflammatory agents (Wang *etal*., 2010), for the treatment and prevention of vascular diseases due to cholesterol-induced atherosclerosis, and as anti-carcinogenic agents (Lauro and Francis, 2000; Xu *et al.,* 2015). Anthocyanins have also been reported to have antiulcer activity and to provide protection against UV radiation (Mazza and Miniati, 1993). Possible mechanisms for the anti-inflammatory activity of anthocyanins include inhibition of arachidonic acid metabolism and the prostaglandin synthase cyclooxygenase activity (Aruoma and Cuppett, 1997). Anthocyanins in red wine may have antiatherogenic effects in conjunction with other polyphenols found in the wine (Frankel, German, Kinsella, Parks, and Kanner, 1993). Kamei *et al*. (1995)Studied the in vitro anticarcinogenic effect of anthocyanins on tumor cells. Glycosides of the aglyconscyanidin and delphinidin have been found to be the most abundant anthocyanins in plants (Meiers *et al*., 2001). Cyanidin lowered serum thiobarbituric acid-reactive substance (TBARS) concentration and increased the oxidation resistance of the serum to lipid peroxidation in rats (Tsuda, Horio, Kitoh, and Osawa, 1999). Delphinidin has been reported to inhibit the growth of human tumor cell line by shutting off the epidermal growth-factor receptor downstream signaling cascade (Tsuda *et al.*, 1999; Zheng *et al*., 2011). Mazza and Miniati and Talcott and Lee (S. T. Talcott and J. H. Lee, 2002) reported the presence of anthocyanidindiglucosides as the anthocyanins present in muscadine grapes.

The objective of this paper was to identify and quantify the anthocyanins present in 10 cultivars of muscadine grapes grown in South Georgia, USA.

2. MATERIALS AND METHODS

Chemicals: Standards (with more than 97% purity) of malvidin-3-O-β-glucopyranoside (mv-3-gl), delphinidin-3-O-β-glucopyranoside (dp-3-glc) petunidin-3-O-β-glucopyranoside(pt-3-glc), peonidin-3-O-β-glucopyranoside (pn-3-glc), and cyanidin-3-3-O-β-glucopyranoside (cy-3-glc) were purchased from Polyphenols Laboratories AS (Sandnes, Norway).

Potassium chloride and sodium acetate were purchased from J.T. Baker Chemical Company (Phillipsburg, NJ). Acetonitrile, methanol, O-phosphoric acid (85 % purity, HPLC grade), formic acid and water (HPLC grade) were purchased from Fisher Scientific (Norcross, GA).

Standards preparation: Stock standard solutions (100 µg/mL.) of the anthocyanins mv-3-gl, dp-3-glc, pt-3-glc, pn-3-glc, and cy-3-glc were prepared with 2 HCl in methanol and stored for a week at -86 °C. Each week new stock solutions were prepared to ensure freshness of the standards. Working standard solutions of 100, 75, 50, and 25 µg/mL were prepared in order to build the calibration curve for each compound using the software TableCurve 2D (Systat Software Inc.). Randomly selected working standards were prepared daily in order to check the performance of the method and for possible degradation of the stock solutions. No degradation of the stock solutions was detected during the week that they were stored.

Samples: Fruits from selected ten muscadine grape cultivars, five bronze (Carlos, Early Fry, Fry, Summit and Late Fry), and five purple (Paulk, Cowart, Supreme, Ison and Noble), grown in South Georgia (USA) and provided by Mr. Jacob Paulk (Paulk Vineyards, Wray, GA) were studied. Fruits were separated into skins, seeds and pulps, and extracted, in triplicate, for the corresponding analysis as described below.

Individual anthocyanins: One gram of each sample was mashed using mortar and pestle to a very fine paste and diluted with 2% HCl in methanol. The samples were vortexed for 1 minute and then placed in a water-bath shaker set at 25 °C and 200 rpm for 24 h. Finally, the samples were vortexed for 1 minute to ensure total extraction. The extracted samples were filtered through a 0.45 µm syringe polypropylene filter and 20 µL aliquot injected into a Hewlett-Packard (Avondale, PA) HP 1100 HPLC system with diode array detector. The mobile phase was: solvent A: O-phosphoric acid/methanol/water (5:10:85, v/v/v), and solvent B: acetonitrile. The gradient for anthocyanin separation is as follows: at 0 min 100 % solvent A, at 5 min 90 % solvent A and 10 % solvent B, and at 25 min 50 % solvent A and 50 %solvent B, with 5 min post-run with HPLC-grade water. Flow rate: 0.5 mL/min. Column: Beckman Ultrasphere Cl8 ODS 4.6 x 250 mm. Temperature: 40 °C. Anthocyanin-3,5-diglucosides were identified and quantified by the chromatographic characteristics of their corresponding anthocyanin-3-monoglucosides, and their identity was verified by mass spectrometric analysis. The mass spectrometric analysis was performed under the same chromatographic conditions described for the HPLC analysis (except that O-phosphoric acid was replaced with formic acid), using a LC-MS system from Thermo Separations Products HPLC instrument (San Jose, California) coupled to a Perkin Elmer Sciex API I plus quadrupole mass spectrometer (Shelton, Connecticut).

Total anthocyanins: Grape parts (skin, seed or pulp) were extracted in 2% HCl in methanol, for 24 h at room temperature in the dark, and diluted to an appropriate concentration with potassium chloride buffer, pH 1, until the absorbance of the sample was within the linear range of a Shimadzu 300 UV-Vis spectrophotometer (Rydalmere, Australia). The spectrophotometer was zeroed with distilled water. Two dilutions of each sample were prepared, one with potassium chloride buffer, pH 1, and the other with sodium acetate buffer, pH 4.5. The dilutions were allowed to equilibrate for 15 min. The absorbance was measured at 520 and at 700 nm (to correct for haze) against a blank cell filled with distilled water, following the pH differential method described by Giusti and Wrolstad(Giusti and Wrolstad, 2001).

Statistics: The statistical analysis was carried out using the Microsoft Excel software package (Microsoft Corporation, Mountain View, CA). The analysis was repeated using three samples and standard deviation recorded. Regression and area under the curve analyses were performed using TableCurve 2.D (Systat Software Inc.).

3. RESULTS AND DISCUSSIONS

Anthocyanins were identified by their retention times and characteristic spectra. Quantification was made using the calibration curves of external standards built for each of the standard compounds (mv-3-gl, dp-3-g1c, pt-3-glc, pn-3-g1c, and cy-3-glc), and their corresponding anthocyanidins, after acid hydrolysis. We found, based on HPLC-MS analysis and in agreement with the findings of S. T. Talcott and J.-H. Lee (2002), that anthocyanin-3,5-diglucosides correlated well to their corresponding anthocynidin. Table-1 shows that delphinidin-3,5-diglucoside (dp-3,5-di-glc) was the most abundant anthocyanin found in muscadine skins ranging from 1.1 to 2.8 mg/100 g fresh weight (FW) in the case of the bronze muscadine skins, and from 23.0 to 95.3 mg/10 g FW for the purple muscadine grape skins. The second most abundant anthocyanin in skins was petunidin 3,5 diglucoside (pt-3,5-di-glc) which was present in all purple skins varying from 19.5 to 52.6 mg/100 g FW, and was detected in three of the five bronze skins ranging from 0.9 to 1.3 mg/100 g FW. Malvidin-3,5-diglucoside (mv-3,5-di-glc), cyanidin-3,5-diglucoside (cy-3,5-di-glc), and peonidin-3,S-diglucoside (pn-3,5-di-glc) were not detected in the skins of the bronze fruits, but they were detected in the purple muscadines ranging from 15.8 to 45.8 mg/100 g FW, from 5.9to 13.8 mg/100 g FW and, from 3.8 to 7.3 mg/100 g FW, respectively.

In addition, petunidin-3-glucoside was the only mono - glucoside detected in the skins of the purple grapes and ranged from 1.3 to 2.3 mg/100 g FW. Delphinidin-3, 5-diglucoside was the only anthocyanin detected in the seeds as well as in the pulps (Table-2).

Table-2 shows the calculated individual anthocyanin content in muscadine grapes.

Table-1. Anthocyanins in Muscadine grape skins (mg/100 g FW) [1].

Cultivar	dp-3,5-di-glc	pt-3,5-di-glc	mv-3,5-di-glc	cy-3,5-di-glc	pn-3,5-di-glc	pt-3-glc
Carlos	1.3 ± 0.1	1.3 ± 0.1	nd	Nd	nd	nd
Early fry	1.4±0.1	1.2 ± 0.1	nd	Nd	nd	nd
Fry	1.1 ± 0.1	nd	nd	Nd	nd	nd
Summit	2.8 ± 0.1	nd	nd	Nd	nd	nd
Late fry	1.2 ± 0.1	0.9 ± 0.1	nd	Nd	nd	nd
Paulk	95.3 ± 4.9	29.8 ± 1.9	45.8 ± 2.8	13.8 ± 0.2	6.3 ± 0.1	2.3 ± 0.1
Cowart	54.9 ± 4.0	28.8 ± 1.9	15.8 ± 0.7	6.0± 0.8	3.7 ± 0.2	1.4 ± 0.1
Supreme	74.9 ± 3.5	33.5 ± 2.1	20.3 ± 1.1	11.5 ± 0.9	3.9± 0.1	1.5 ± 0.1
Ison	85.1 ± 4.0	52.6 ± 2.9	31.5 ± 1.2	9.3 ± 0.9	7.3 ±0.2	2.3 ± 0.2
Noble	23.0 ± 2.0	19.5 ± 1.0	31.9 ± 1.5	5.9 ± 0.5	3.8 ± 0.1	1.3 ± 0.1

1 Values are average and standard error of triplicates; nd = not detected

Table-2. Delphinidin-3,5-diglucoside in seeds and pulps (mg/100 g FW)[2].

Cultivar	Seeds	Pulps
Carlos	1.3±0.1	1.3±0.1
Early fry	1.4±0.1	1.2±0.1
Fry	1.1±0.1	Nd
Summit	2.8±0.1	Nd
Late fry	1.2±0.1	0.9±0.1
Paulk	95.3±4.9	29.8±1.9
Cowart	54.9±4.0	28.8±1.9
Supreme	74.9±3.5	33.5±2.1
Ison	85.1±4.0	52.6±2.9
Noble	23.0±2.0	19.5±1.0

2 Values are average and standard error of triplicates; nd = not detected

It was found in the seeds of four of the five bronze fruits and ranged between 3.1 to 5.8 mg/100 g FW, and also detected in all the seeds of the 5 purple fruits with concentration varying from 2.4 to 6.4 mg/100 g FW. Delphinidin-3, 5-diglucoside was not detected in the pulps of the bronze fruits, but detected in 3 of the 5 pulps of purple fruits with concentrations ranging from 1.7 to 4.2 mg/100 g FW. Table-3 shows the calculated individual anthocyanin content in muscadine grapes.

The calculation was based on the anthocyanin content in each fruit part multiplied by the weight fraction of the fruit part to the weight of the whole fruit (data not shown). The anthocyanin profile in the whole grapes followed the same trend as the skins because skins are a major component of the fruit (41.2% of the whole fruit, on average). Skins account for the majority of the pigment content in the grape. The total anthocyanin content of muscadine grape parts (Table-4) varied from 0.4 to 1.3 mg/100 g FW for the bronze skin grapes and from 31.1 to 74.5 mg/100 g FW in the case of the purple grapes.

On average, 92.4% of the total anthocyanin content was found in the skins of the grapes, 6.1% in the seed and 1.5% in the pulps. The low percentage of anthocyanins in pulps may be due to some transfer of anthocyanins from the skin at the moment of the separation because most pulps were basically colorless. Anthocyanin content in muscadine grapes was found to be lower compared to other grapes or berries; however, this comparison is not reliable due to the different methods that each author reported for extraction and analysis.

Our results are in agreement with those published by Mazza and Miniati (1993) and Goldy, Maness, Stiles, Clark, and Wilson (1989)who reported the same order in the concentration of anthocyanin- diglucosides in muscadine grapes with delphinidin-3,5-diglucoside as the major anthocyanin. However, there was no agreement in the proportions of the individual anthocyanins, which is not surprising because agro-ecological and varietal factors may affect the morphological characteristics and the chemical composition of agricultural products. In addition to the 5 diglucosides reported by Mazza and Miniati (1993), we were able to identify a monoglucoside (petunidin-3-glucoside). Figure-1 shows the HPLC chromatograms for A: Monoglucosidesstandards and B: muscadine grape skin sample.

Figure-2 shows the mass spectra for a grape skin sample showing the different molecular ions for the anthocyanins present.

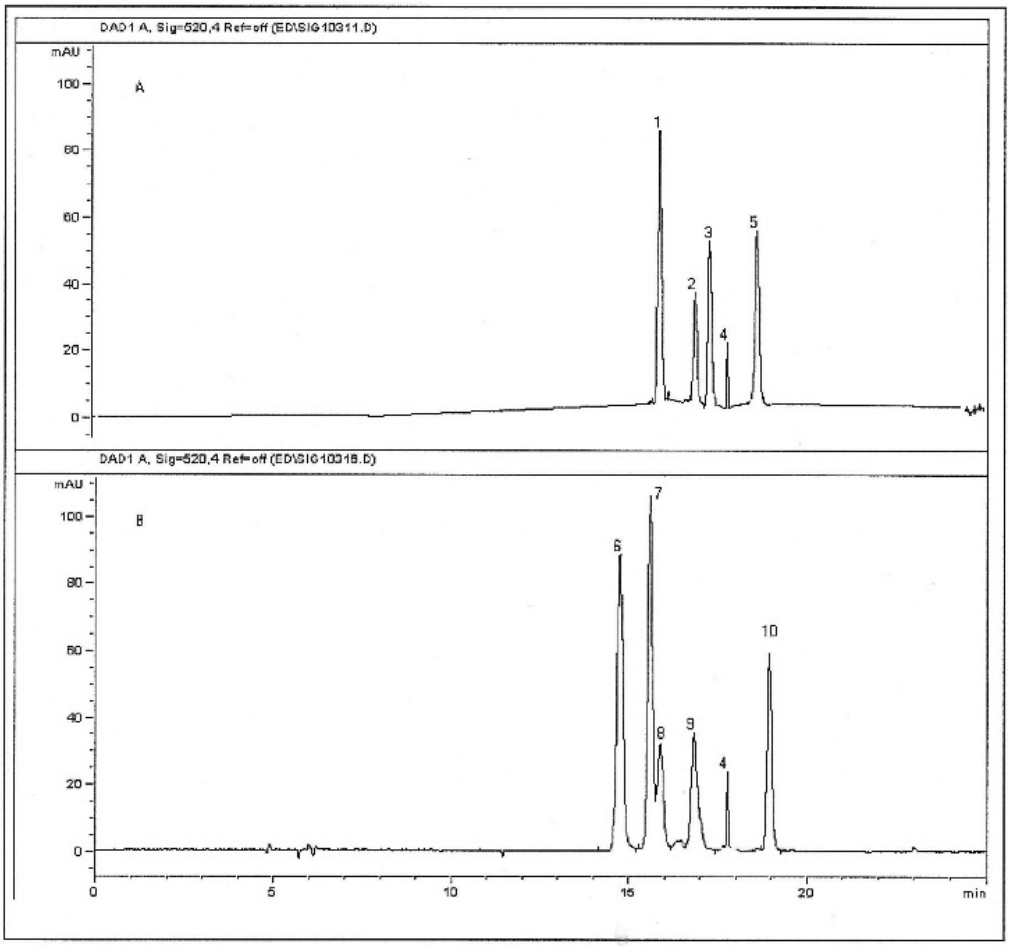

Figure-1. (A) HPLC chromatrogram at 520 nm of anthocyanin monoglucosides standards: delphinidin-3-glucoside (1), cyanidin-3-glucoside (2), petunidin-3-glucoside (3), peonidin-3-glucoside (4), malvidin-3-glucoside (5). **(B)** HPLC chromatrogram at 520 nm of the anthocyanins found in the skin of the grapes of the cultivar paulk: deplphinidin-3,5-diglucoside (6), cyanidin-3,5-diglucoside (7), petunidin-3,5-diclucoside (8), malvidin-3,5-diglucoside (9), peonidin-3,5-diglucoside (10).

Table-3. Anthocyanins in Muscadine grapes (mg/100 g FW)[3].

Cultivar	dp-3,5-di-glc	pt-3,5-di-glc	mv-3,5-di-glc	cy-3,5-di-glc	pn-3,5-di-glc	pt-3-glc	Total
Carlos	0.4	0.4	nd	nd	nd	nd	0.8
Early fry	0.6	0.4	nd	nd	nd	nd	1.0
Fry	0.6	nd	nd	nd	nd	nd	0.6
Summit	1.3	nd	nd	nd	nd	nd	1.3
Late fry	0.7	0.4	nd	nd	nd	nd	1.1
Paulk	41.3	12.1	18.2	5.6	2.6	2.3	82.1
Cowart	19.0	9.8	5.4	2.0	1.3	1.4	38.9
Supreme	35.9	16.0	9.7	5.5	1.8	1.5	70.4
Ison	34.4	20.6	12.3	3.7	2.8	2.3	76.1
Noble	11.8	9.0	14.8	2.7	1.7	1.3	41.3

3 Values are calculated based on the weight of each pat to the whole fruit; nd = not detected

Table-4. Total Anthocyanins in Muscadine grape parts and whole fruit (mg/100 g FW as cyanidin-3-glucoside)[4].

Cultivar	Skin	Seed	Pulp	Whole fruit
Carlos	2.5	1,3	nd	0.9
Early fry	2.5	8.5	nd	1.1
Fry	0.7	4.1	nd	0.4
Summit	2.9	3.5	nd	1.3
Late fry	2.0	4.0	nd	1.1
Paulk	174.5	4.0	4.2	74.5
Cowart	101.5	4.4	0.9	37.5
Supreme	143.2	7.8	0.8	65.6
Ison	170.2	4.1	1.7	69.4
Noble	67.8	2.1	2.1	31.1

4 Values are average of triplicates; nd = not detected

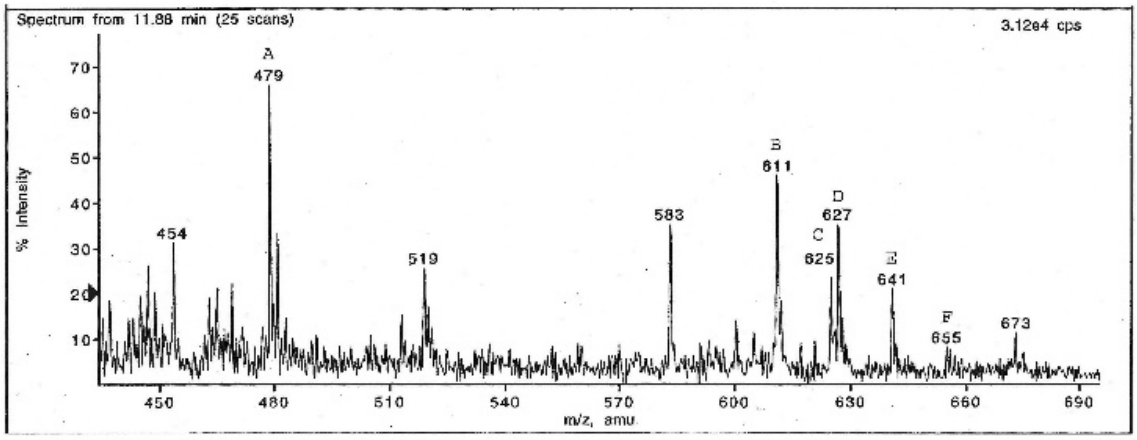

Figure-2. Positive mass spectrum of the molecular ions found in a skin sample of the Paulk cultivar. Petunidin-3-glucoside (A), cyanidin-3,5-diglucoside (B), peonidin-3,5-diglucoside (C), delphinidin-3,5-diglucoside (D), petunidin-3,5-diglucoside (E), and malvidin-3,5-diglucoside (F). Other peaks were not identified.

As indicated above, quantification of anthocyanin diglucosides was based on the calibration curves built for the monoglucosides standards and their corresponding anthocyanidins, following the method described by S. T. Talcott and J.-H. Lee (2002).

The anthocyanin profile of muscadine grapes differ greatly from the European grapes *(Vitisvinifera).* The anthocyanin profile is more complex in the case of the *Vitisvinifera*grapes which have more than 20 different anthocyanins with the major one being malvidin-3-glucoside (J. P. Goiffon, M. Brun, and M. J. Bourrier, 1991). All anthocyanins in *Vitisvinifera*are monoglucosides, some are acylated, with no diglucosides present (Mazza, Fukumoto, Delaquis, Girard, and Ewert, 1999; Mazza and Miniati, 1993). In contrast, muscadine grapes *(Vitisrotundifolia)* have mainly anthocyanin diglucosides, none of the anthocyanins is acylated and malvidin is a minor pigment in this kind of grapes. The differences in the pattern of anthocyanins may be due to the different evolutionary paths taken by the two species. In addition, European grapes have a long history of artificial breeding aimed at the improvement of wine quality and at the search for new tastes, colors and aromas. In contrast, muscadine grapes have remained close to their natural state, subjected to natural selection, and for just a few decades subjected to selective breeding.

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