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Identification of anthocyanins in muscadine grapes with HPLC-ESI-MS

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ABSTRACT

Muscadine grapes are important fruits grown in the southeastern United States. They are a good source of anthocyanins, but confirmatory analyses of the structure of individual anthocyanins in muscadine grapes using more advanced instrument are lacking. In this study, the anthocyanins of muscadine grapes were analyzed using HPLC-MS with electrospray ionization. Approximately 90% of the total anthocyanins were 3,5-diglucoside of delphinidin, cyanidin and petunidin; the remaining 10% were 3,5-diglucoside of peonidin and malvidin. There was a significant difference of total anthocyanin content among different cultivars of muscadine grapes with purple-skinned muscadine grapes having significantly higher levels of anthocyanins than bronze-skinned muscadine grapes. The total anthocyanin content in purple-skinned Jumbo and Cowart muscadine was 4.1 and 2.6 mg/g dry weight, respectively, while the total anthocyanin content in bronze-skinned Higgin and Carlos was negligible. The anthocyanins were mainly concentrated in the skin of the grapes. The results of the present study confirmed some of the anthocyanins reported by previous studies and also found one anthocyanin that might be falsely identified by previous studies.

1. Introduction

Muscadine grapes (*Vitis rotundifolia*) are native to the southeastern United States. The climate and soil in this region make it an ideal place for muscadine cultivation. Due to the humid summer and warm winter, traditional *Vitis* species, the so-called bunch grapes or wine grapes are not well adapted to this region. Muscadine grapes are also resistant to Pierce's disease and other pests which are detrimental to other grape species (Olien & Hegwood, 1990). The unique aroma and flavor characteristics make muscadine grapes a widely welcomed fruit among consumers in this region. Muscadine grapes can be served fresh, processed into juice, wine, jam, and other products. Cultivation of muscadine provides a tremendous potential for local farmers to increase their income. Studies have demonstrated that muscadine grapes are rich sources of bioactive phytochemicals that are associated with the prevention of chronic diseases such as cardiovascular diseases and cancer (Pastrana-Bonilla, Akoh, Sellappan, & Krewer, 2003). The phytochemicals in muscadine grapes include gallic acid, catechin, epicatechin, ellagic acid, myricetin, quercetin, kaempferol, resveratrol, and anthocyanins (Lee, Johnson, & Talcott, 2005; Pastrana-Bonilla, et al., 2003). Muscadine grapes are also an excellent source of dietary fiber. As consumers become aware of these health benefits associated with the consumption of muscadine grapes, demand for fresh and processed products from muscadine grapes has increased. As a result, the muscadine grape industry is currently experiencing its greatest growth in decades and the outlook for continued future expansion is excellent (Striegler et al., 2005).

Anthocyanins are a group of pigments in plants. They are responsible for the brilliant color (red, blue, or purple) of flowers, fruits, and vegetables. The anthocyanin molecule is composed of an aglycone anthocyanidin and some sugar moieties. The common anthocyanidins found in plants are Cyanidin (Cy), Delphinidin (Dp), Petunidin (Pt), Peonidin (Pn), Malvidin (Mv), and Pelargonidin (Pg) (Andersen & Jordheim, 2006). One or more sugar molecules can be linked to anthocyanidin through a glycosidic bond. It was reported that there are more than 600 types of anthocyanins in nature. The structure of these anthocyanins differs in the types of anthocyanidins, sugar molecules and numbers, and types of acylation groups. Due to their bright color and high water solubility, anthocyanins are considered a potential natural pigment to replace artificial food colorants (Mazza & Miniati, 1993). Besides the coloring functions, anthocyanins in food also possess potent antioxidant capacity and health promoting properties. For instance, anthocyanins are believed to reduce the risk of cardiovascular diseases for people who consume wine, berry, and grape. The mechanism is postulated as that anthocyanins act as antioxidants by donating hydrogen atoms to highly reactive free radicals, breaking the free radical chain reaction (Rice-Evans, Miller, & Paganga, 1996). Other reports found significant anti-inflammatory properties in both *in vitro* and *in vivo* studies on the bioefficacy of

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powder prepared from muscadine grape skins (Greenspan et al., 2005). One of the finding was that anthocyanins down-regulated the pro-inflammatory cytokines in an asthma model of murine (Park, Shin, Seo, & Kim, 2007). These findings indicated that consumption of foods rich in anthocyanins might promote the well-being and prevent certain diseases for the public, including cardiovascular diseases and some inflammatory diseases.

Although several previous studies have quantified the total anthocyanin content in muscadine grapes (Pastrana-Bonilla, et al., 2003), studies on individual anthocyanins, especially the chemical structure of anthocyanins in muscadine grapes are still incomplete. Since anthocyanins are important to the quality and health benefit of muscadine grape products, it would be critical to elucidate the actual types of anthocyanins in muscadine grapes. Identification of anthocyanin is challenging because of their complex structure and the lack of reference compounds for comparison. Earlier studies used thin layer chromatography or HPLC coupled with a photodiode array detector (PDA) to analyze individual anthocyanins (Bakker et al., 1997; Garcia-Viguera, Zafrilla, & Tomas-Barberan, 1997; Morais, Ramos, Forgacs, Cserati, & Oliviera, 2002). With these techniques, compound identification was based primarily on the UV–visible spectrum or retention time as compared with the standard compounds. Since the UV–Vis detector cannot differentiate the co-eluted compounds, the structure information and compound identification capacity provided by these methods are very limited. Therefore, confirmatory analyses using more advanced instrumentation are needed. HPLC coupled with electrospray ionization (ESI) mass spectrometer (MS), especially the tandem mass spectrometer can provide mass spectrum of intact molecular ion and fragment ions. The peaks can be identified categorically by matching their mass spectrum and retention time with reference compounds. Obviously, the HPLC-MS should be the method of choice for the analysis of anthocyanins. However, we have not found any reported studies on the structure of anthocyanins in muscadine grapes using HPLC-MS. The structural information generated from HPLC-ESI-MS method may allow us to verify the results of anthocyanins identified in muscadine grapes by previous studies and the information will also lead to a better understanding of the health benefits of muscadine products. Therefore, the objective of the present study was to identify and quantify individual anthocyanins in different cultivars of muscadine grapes using the HPLC-ESI-MS method.

2. Material and methods

2.1. Standard and chemicals

An anthocyanidin standard kit containing cyanidin chloride, delphinidin chloride, pelargonidin chloride, peonidin chloride, and malvidin chloride was purchased from Cerilliant Corporation (Round Rock, TX). Petunidin chloride was obtained from ChromaDex™ Corporation (Irvine, CA). Formic acid, hydrochloric acid and all other solvents were purchased from Fisher Scientific (Fair Lawn, NJ).

2.2. Sample collection

Muscadine grapes were picked from the farm of the George Washington Carver Agricultural Experiment Station at Tuskegee University. The muscadine grapes were field grown using conventional agricultural practice. Four cultivars were collected with two bronze varieties of Higgins and Carlos and two purple varieties of Jumbo and Cowart. After collection, approximately 200 g berry of each cultivar was frozen at −20°C and then freeze-dried. Half of the dried fruit was ground into powder for analysis of anthocyanins in whole fruit; the other half was separated into skins, seeds and pulp before they were ground into powder separately for the analysis of anthocyanin content in different parts of the fruit.

2.3. Anthocyanin extraction

Anthocyanins were extracted in methanol solution. About 1 g of ground samples and 50 mL 80:20 (v/v) methanol–water solution containing 0.1 mL/L HCl were added to a tube with a screw cap. Nitrogen was used to flush the air out of the tube. The mixture (total volume 50 mL) was placed in a shaking water-bath (Gyrotory® Model G76, New Brunswick Scientific, Edison, NJ) at 30 °C in the dark for 16 h (Hakkinen et al., 1999). After sonication for 15 min, the samples were centrifuged at 2000 g for 10 min. An aliquot of 10 mL supernatant was added to a rotary evaporator to evaporate the solvent under vacuum at 40 °C. The residual was resuspended in acidified water containing 0.1 mL/L HCl. A Waters C_{18} Sep-Pak® solid phase extraction cartridge (Milford, MA) was pre-conditioned with 5 mL methanol and 5 mL 0.1 mL/L HCl acidified water before use, and then the suspension was loaded to the pre-conditioned cartridge. The cartridge was washed with acidified water. Anthocyanins were eluted with 10 mL methanol. The elute was used to analyze the total anthocyanin content. For individual anthocyanin identification, the elute was filtered through a 0.2 μm nylon syringe filter and analyzed by HPLC-ESI-MS.

2.4. Total anthocyanins content measurement

Total anthocyanin content in muscadine grapes was measured using the pH differential spectrophotometric method described by Giusti and Wrolstad (2001). The anthocyanin extract was dissolved in 0.025 mol/L potassium chloride buffer, pH 1.0 and 0.4 mol/L sodium acetate buffer, pH 4.5 with a pre-determined dilution factor. The absorbance was measured at 520 nm and 700 nm. The absorbance (A) of the diluted sample was then calculated as follows:

$$A = (A_{520} - A_{700})_{\text{pH 1.0}} - (A_{520} - A_{700})_{\text{pH 4.5}}$$

The monomeric anthocyanin concentration in the original sample was calculated in cyanidin-3,5-diglucoside equivalents according to the following formula:

$$\text{Anthocyanin content} = \frac{A \times \text{MW} \times \text{DF} \times 1000}{\varepsilon \times 1}$$

where MW (611) of cyanidin-3,5-diglucoside is used because the anthocyanin content was calculated in cyanidin-3,5-digulcoside equivalent; the molar absorptivity ε was 30,175; the DF was dilution factor; 1000 is the factor to convert gram to milligram; the A was absorbance. Anthocyanin content was mg/L which was then converted to mg/g dried sample.

2.5. HPLC analysis of anthocyanins

The HPLC-ESI-MS system was composed of Shimadzu LC-20AD HPLC system, Shimadzu SPD-20AV UV–Vis detector, and Shimadzu 2010EV mass spectrometer coupled with an electrospray interface (Shimadzu, Kyoto, Japan). The column was Symmetry C_{18} with 5 μm particle size, 150 mm × 3.0 mm (Waters Corporation, Milford, MA). The mobile phase was 5:95 (v/v) formic acid–water solution (phase A) and 5:95 (v/v) formic acid–methanol solution (phase B) at a flow rate of 0.8 mL/min. The linear gradient of phase B was 15% for the first 2 min, increased from 15% to 45% from 2 min to 30 min, and decreased from 45% to 15% from 30 min to 30.1 min. Finally, isocratic elution with 15% phase B was maintained until 40 min. UV–Vis detector wavelength was set at 520 nm. Mass spectra were acquired in positive ion mode. Ion was