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# Nutraceutical Changes in Muscadine Grape and Grape Segments During Storage

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## Abstract

Fresh-market muscadine (*Vitis rotundifolia* Michx.) cultivars, such as 'Supreme', have reportedly improved postharvest storability. Physiochemical attributes and nutraceutical compounds in 'Supreme' muscadine whole grape berries and grape segments (flesh [skin and pulp] and seeds) were measured at harvest in 2012 and 2013 and nutraceutical compounds were measured during postharvest storage for 6 weeks at 2°C in 2012. Total anthocyanins, ellagitannins, and flavonols in whole berries and flesh were higher than seeds, while total phenolics and Oxygen Radical Absorbance Capacity (ORAC) were higher in whole berries than the flesh and seeds. Anthocyanins were present in the whole berries and the flesh, but not in the seeds. Total ellagitannins were distributed with 81% in flesh and 19% in seeds. Total flavonols were found 90% in flesh and 10% in seeds and total phenolics were present 55% in flesh and 45% in seeds. In 2012, 48% of ORAC was accounted for in flesh and 52% in seeds, conversely in 2013, 63% was in flesh and 37% in seeds. Resveratrol was almost completely found in flesh (96%) with the remaining 4% in seeds. Postharvest storage did not dramatically impact nutraceutical components in the whole berries or the segments. Total anthocyanins were negatively correlated with total ellagitannins ($r = -0.94$) and total flavonols ($r = -0.88$). Total ellagitannins were positively correlated with total flavonols ($r = 0.97$). ORAC was positively correlated with total phenolics ($r = 0.88$). Both the discovery that postharvest storage of muscadine grapes did not dramatically impact nutraceutical components in the whole berries or the segments and the identification and quantification of nutraceutical components in berry tissues provides additional information on nutraceuticals in this underutilized fruit.

Muscadine grapes (*Vitis rotundifolia* Michx.) are indigenous to the southeastern United States and have been cultivated for over 400 years (Conner, 2009). Muscadine grapevines are an important *Vitis* species (Marshall and Stringer, 2014) partially due to their high level of disease resistance. This native grape is grown in small to large vineyards as well as home plantings, ranging from North Carolina and Florida to eastern Oklahoma and Texas. Muscadine grape berries vary in color (from bronze to black), shape, and size, but are typically sweet with a unique fruity flavor. The berries usually have thick skins and contain 3-4 seeds per berry.

Consumer demand for muscadines and muscadine products has increased since muscadines have been shown to have high levels of nutraceutical compounds such as flavonols, ellagic acid derivatives, anthocyanins, and phenolics, which result in high antioxidant capacity (Ector et al., 1996; Lee and Talcott, 2004; Marshall et al., 2012; Perkins-Veazie et al., 2012; Sandhu and Gu, 2010; Shi et al., 2003; Threlfall et al., 2005). Muscadines have traditionally been used for the production of juice, wine, jams, and jellies, but interest has expanded for commercial fresh-market fruit production. Major limiting factors in fresh-market muscadine commercialization expansion are uneven ripening, short harvest season, seediness, firmness, and postharvest storability (Conner, 2009; Perkins-Veazie et al., 2012), but

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these factors are being addressed by breeding programs to develop improved cultivars along with other research in production and postharvest handling.

Studies have been conducted to quantify nutraceutical compounds in muscadines, muscadine products, and in different muscadine berry segments (skins, pulp, and seeds) (Lee and Talcott, 2004; Marshall et al., 2012; Sandhu and Gu, 2010; Shi et al., 2003; Takeda et al., 1983; Threlfall et al., 2005). However, there is limited information on the impact of extended postharvest storage on nutraceutical compounds in muscadines. The objective of this study was to identify nutraceutical compounds in 'Supreme' muscadine whole grape berries and grape segments (flesh [skin and pulp] and seeds) and determine how the nutraceutical compounds were affected by postharvest storage.

## Materials and Methods

*Vineyard*

Muscadine grapevines were grown at the University of Arkansas Fruit Research Station, Clarksville, AR (lat. 35°31'58'N and long. 93°24'12'W), in Linker fine sandy loam, in USDA hardiness zone 7a, where average annual minimum temperature reached −15°C. Vines were spaced 6.1 m apart with rows spaced 3.0 m apart. Vines were trained to a bilateral cordon on a single-wire trellis. The vines were dormant-pruned annually in February using spur pruning with spurs retained of two to four buds in length. Weeds were controlled with pre- and post-emergence herbicides as needed, and vines did not have any stress from weed competition. Vines were drip irrigated as needed. Vines were nitrogen fertilized annually in March at a rate of ≈70 kg·ha$^{-1}$. No insecticides, fungicides or other pest control compounds were applied to the vines**.** The vines produced full crops during the study, and there was no crop reduction due to winter injury or other limitations. Daily maximum and minimum temperatures and rainfall were recorded (data not shown).

*Experimental design*

In 2012, composition and nutraceutical compounds were measured initially (immediately after harvest) and during postharvest storage weekly for 6 weeks at 2°C. In 2013, composition and nutraceutical compounds were measured after harvest only. Samples in 2012 and 2013 were taken in triplicate from each vine in a three-vine plot. The composition and nutraceutical components were analyzed initially using nine berries/replication (three berries for composition, three whole berries for nutraceuticals, three segmented berries for nutraceuticals). In 2012, nutraceutical components were measured during postharvest storage every 7 d (three whole berries for nutraceuticals and three segmented berries for nutraceuticals). In 2012, the experimental design was a split-split-plot; the first split was postharvest storage (0, 1, 2, 3, 4, 5, and 6 weeks) and the second split was berry segment (flesh [skin and pulp], seed, and whole berry). The data for both 2012 and 2013 were also analyzed as split-split-plot; the first split was year (2012 and 2013) and the second split was berry segment.

*Cultivar, harvest, and transport*

The muscadine cultivar 'Supreme' was used in this study since it is a prominent black cultivar in the commercial industry and has the potential for extended postharvest storage (Conner, 2013). In both years, vines were once-over, hand-harvested late in the afternoon. Harvest date was based on soluble solids concentration, ease of release from the pedicel, and berry color. The muscadines were transported to University of Arkansas Institute of Food Science and Engineering, Fayetteville, AR, on the same day of harvest and placed in cold storage (2°C) upon arrival. The muscadines were randomly selected for analysis.

*Composition analysis*

For both years, composition was measured from whole berries at harvest, using juice strained through cheesecloth to remove any

solids. Composition measurements included soluble solids concentration (%), pH, and titratable acidity (TA) (%). Titratable acidity and pH were measured by an 877 Titrino Plus (Metrohm AG, Herisau, Switzerland) with an automated titrimeter and electrode standardized to pH 2.0, 4.0, 7.0, and 10.0 buffers. Titratable acidity was determined using 6 g of juice diluted with 50 mL of deionized, degassed water by titration of 0.1 N sodium hydroxide (NaOH) to an endpoint of pH 8.2, and results were expressed as percent tartaric acid. Soluble solids were measured using a Bausch and Lomb Inc. Abbe Mark II refractometer (Rochester, NY).

*Postharvest storage*

For postharvest storage in 2012, berries were hand-sorted to remove any split, shriveled, or decayed fruit before packaging. As with a commercial product, only sound, marketable, fruit were used. The berries were packed into hinged standard vented clamshells (18.4 cm x 12.1 cm x 8.9 cm) (H116, FormTex Plastics Corporation, Houston, TX) and stored at 2°C with 85-90% relative humidity (RH). From the harvested berries, three clamshell containers (three replications) were filled to approximately 500 g. Every 7 d for 6 weeks, six randomly selected berries from each replication were removed, placed in plastic bags, and stored at -20°C until analysis.

*Nutraceutical analysis*

For nutraceutical analysis, the bags of frozen berries were placed in 30°C water for 30 s, to allow berries to thaw. Three whole berries were used for analysis, and another three berries were divided into flesh (skin and pulp) and seeds for analysis. The segmented berries were cut longitudinally, and the seeds were removed from the flesh. The whole berries, flesh, and seed segments for each replication were prepared separately for nutraceutical analysis. Samples were homogenized using a Euro Turrax T18 Tissuemizer (Tekmar-Dohrman Corp, Mason, OH) for 1 min with alternating washes of extraction solution containing methanol/water/formic acid (MWF) (60:37:3 v/v/v) and acetone/water/acetic (70:29.5:0.5 v/v/v). Homogenates were centrifuged for 5 min at 10,000 rpm and filtered through Miracloth (CalBiochem, La-Jolla, CA). The samples were adjusted to a final volume with extraction solvent and stored at -70°C until further analysis. Prior to high performance liquid chromatography (HPLC) analysis, the samples were filtered through 0.45 µm filters (Whatman PLC, Maidstone, UK). Nutraceutical concentrations and antioxidant capacity evaluations were performed using methods described in Cho et al. (2004; 2005), Hager et al. (2008), Lee et al. (2005), and Prior et al. (2003).

*Total phenolics analysis*. Total phenolics were measured using the Folin-Ciocalteu assay (Slinkard and Singleton, 1977) on a diode array spectrophotometer (8452A; Hewlett Packard, Palo Alto, CA), with a gallic acid standard and a consistent standard curve based on sequential dilutions. Samples were prepared with 1 ml 0.2N Folins reagent, 0.8 ml $Na_2CO_3$ (75 $g \cdot L^{-1}$) and 0.2 mL of extracted sample with a reaction time of 2 h. Absorbance was measured at 760 nm, and results were expressed as mg of gallic acid equivalents (GAE) per 100 g fresh weight.

*Anthocyanin, ellagitannin, flavonol, and resveratrol analysis.* For anthocyanin, ellagitannin, and flavonol analysis, subsamples (5 mL) of supernatant were evaporated to dryness using a SpeedVac® concentrator (ThermoSavant, Holbrook, NY) with no radiant heat applied and suspended in 1 mL of aqueous 3% formic acid solution. Samples (1 mL) were analyzed using a Waters HPLC system equipped with a model 600 pump, a model 717 Plus autosampler, and a model 996 photodiode array detector. Separation was carried out using a 4.6 mm × 250 mm Symmetry® C18 column (Waters Corp, Milford, MA) preceded by a 3.9 mm × 20 mm Symmetry® C18 guard column. The mobile phase was a linear gradient of 5% formic acid and methanol from 2% to 60% for 60

min at 1 mL·min$^{-1}$. Prior to each injection, the system was equilibrated for 20 min at the initial gradient. Detection wavelength was 510 nm for anthocyanins. Individual anthocyanin diglycosides were quantified as cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), and malvidin (Mv) glycoside equivalents using external calibration curves containing a mixture of authentic standards (Polyphenols, Sandnes, Norway). Total anthocyanins were calculated as the sum of individual glycosides.

For total flavonol, total ellagitannin, and resveratrol analysis, samples (5 mL) of supernatant were evaporated to dryness using a SpeedVac® concentrator with no radiant heat applied and suspended in 1 mL of aqueous 50% methanol solution. The samples were analyzed using the same HPLC system described above. Separation was carried out using a 4.6 mm × 250 mm Aqua® C18 column (Phenomenex, Torrance, CA) preceded by a 3.0 mm × 4.0 mm ODS® C18 guard column (Phenomenex). The mobile phase was a gradient of 20 g·kg$^{-1}$ acetic acid (A) and 5 g·kg$^{-1}$ acetic acid in water and acetonitrile (50:50 v/v, B) from 10% B to 55% B in 50 min and from 55% B to 100% B in 10 min. Prior to each injection, the system was equilibrated for 20 min at the initial gradient. A detection wavelength of 360 nm was used for flavonols, 280 nm for ellagitannins, and 220 nm for resveratrol at a flow rate of 1 mL·min$^{-1}$. Flavonols and ellagitannins were expressed as mg of rutin equivalents 100 g$^{-1}$ fresh weight, and mg of ellagic acid equivalents 100 g$^{-1}$ fresh weight, respectively. Resveratrol (3,4′,5-Trihydroxy-*trans*-stilbene,5-[(1*E*)-2-(4-Hydroxyphenyl)ethenyl]-1,3-benzenediol) was quantified using external calibration curves of an analytical standard (Sigma-Aldrich Co. LLC, St. Louis, MO), with results expressed as mg per 100 g$^{-1}$ fresh weight.

*High Performance Liquid Chromatography/Mass Spectrometry (HPLC/MS).* For HPLC/MS analysis the HPLC apparatus was interfaced to a Bruker Esquire (Bruker Corporation, Billerica, MA) LC/MS ion trap mass spectrometer. Mass spectral data were collected with the Bruker software, which also controlled the instrument and collected the signal at 280 or 360 nm. Typical conditions for mass spectral analysis in negative ion electrospray mode included a capillary voltage of 4000 V, a nebulizing pressure of 30.0 psi, a drying gas flow of 9.0 mL·min$^{-1}$ and a temperature of 300°C. Data were collected in full-scan mode over a mass range of $m/z$ 50 – 1000 at 1.0 s/cycle. Characteristic ions were used for peak assignment, with results compared with previous mass to charge ($m/z$) values reported for flavonols (Sandhu and Gu, 2010) and ellagic acid derivatives (Lee et al., 2005; Sandhu and Gu, 2010) in muscadines.

*Oxygen Radical Absorbance Capacity (ORAC) analysis.* The ORAC of muscadine extracts was measured using the method of Prior et al. (2003) modified for use with a FLUOstar Optima microplate reader (BMG Labtechnologies, Durham, NC) using fluorescein as fluorescent probe. Muscadine extracts were diluted 1600-fold with phosphate buffer (75 mM, pH 7) prior to ORAC analysis. The assay was carried out in clear 48-well Falcon plates (VWR, St. Louis, MO), each well having a final volume of 590 µL. Initially, 40 µL of diluted sample, Trolox equivalants (TE) standards (6.25, 12.5, 25, 50 µM) and blank solution of phosphate buffer were added to each well. The FLUOstar Optima instrument equipped with two automated injectors was programmed to add 400 µL of fluorescein (0.108 µM) followed by 150 µL of 2,2'-azobis(2-amidino-propane) dihydrochloride (AAPH) (31.6 mM) to each well. Fluorescence readings (excitation 485 nm, emission 520 nm) were recorded after the addition of fluorescein and AAPH and every 192 s for 112 min to reach 95% loss of fluorescence. Results were based upon differences in areas under the fluorescein decay curve between the blank, samples, and standards, and expressed relative to the initial reading. The standard curve was obtained by plotting the four concentrations of TE against the net area under the curve of

each standard. Final ORAC values were calculated using the regression equation between TE concentration and the net area under the curve and expressed as μmol TE equivalents per g fresh weight.

*Statistical analysis*

Prior to analysis, the berry flesh and seed data were adjusted to represent the proportion of the total berry weight. This was done by determining the percent seed and flesh weight of the total berry weight and then multiplying the nutraceutical concentrations by this value (≈ 2 and 98%, respectively). The data were analyzed by analysis of variance (ANOVA) using JMP® (version 11.0; SAS Institute Inc., Cary, NC). Tukey's Honest Significant Difference was used for mean separations ($p$ = 0.05). Associations among all dependent variables were determined using multivariate pairwise correlation coefficients of the mean values using JMP® (version 11.0; SAS Institute Inc., Cary, NC).

## Results and Discussion

*Composition and nutraceutical compounds by year*

Substantial differences were observed in minimum and maximum temperatures as well as for precipitation between 2012 and 2013. During the growing and harvest seasons (April through September) differences of temperatures up to 5°C warmer, and approximately half as much precipitation was observed in 2012 compared to 2013. These extreme differences in weather between years of the study offered some important insight on the significance of environment on composition and nutraceutical content of muscadine grapes.

At harvest in 2012 and 2013, the soluble solids of the muscadines were 19.5 and 14.7%, pH values were 3.8 and 4.0, and the TAs were 0.23% and 0.17%, respectively. For years, the *F*-test indicated significant interactions of the initial nutraceutical compounds between year and berry segments for total anthocyanins (P = 0.0432), total ellagitannins (P = 0.0288), total flavonols (P = 0.0256), and ORAC (P = 0.0001). As expected, year had a dramatic effect on nutraceutical compounds as the temperature differences between years were substantial. The nutraceutical compounds identified are presented in Table 1.

*Anthocyanins.* Anthocyanins were found in the flesh of the whole berries, but not in seeds. Anthocyanin concentrations were overall higher in 2012 compared to 2013 (Table 1). Total anthocyanins found in whole berries of 'Supreme' were lower than those reported by Conner and MacLean (2013), Pastrana-Bonilla et al. (2003), Striegler et al. (2005), and Yi et al. (2005). Additionally, Pastrana-Bonilla et al. (2003) and Striegler et al. (2005) identified anthocyanins in berry seeds of 'Supreme', and Threlfall et al. (2005) found anthocyanins in the seeds of 'Black Beauty', which is contrary to the findings of our study. Total anthocyanins were negatively correlated with total ellagitannins (r = −0.94) and total flavonols (r = −0.88).

*Ellagitannins.* Total ellagitannins were highest in the flesh and whole berries both years of the study compared to seeds (Table 1). For total ellagitannin content, 76% was found in the flesh and 24% was in the seeds in 2012, while in 2013, 87% was in the flesh and 13% was in the seeds. No differences among berry segments were found across years of the study. Total ellagitannins were positively correlated with total flavonols (r = 0.97).

*Flavonols.* The total flavonol concentrations of 'Supreme' are widely unstudied; however, total flavonol concentrations were lower than those reported by Marshall et al. (2012) and Talcott and Lee (2002) for other muscadine cultivars. For total flavonol content, 90% was found in the flesh and 10% was located in the seed both years of the study. Similar to our findings, Sandhu and Gu (2010) found total flavonols to be highest in the muscadine skin and pulp compared to seed (Table 1). Minimal differences were found in flavonol concentrations between years of study (Table 1).

**Table 1.** Nutraceutical compounds in 'Supreme' muscadine (*Vitis rotundifolia* Michx.) whole berries and berry segments at harvest (2012 and 2013).

| Year | Fruit | Total anthocyanins (mg 100 g$^{-1}$) | Total ellagitannins (mg 100 g$^{-1}$) | Total flavonols (mg 100 g$^{-1}$) | Total phenolics (mg 100 g$^{-1}$) | ORAC (µmol TE·g$^{-1}$)[x] | Resveratrol (mg·100 g$^{-1}$) |
|---|---|---|---|---|---|---|---|
| 2012 | Whole | 23.9 a A[z] | 2.9 a AB | 8.7 a AB | 368.7 a A | 66.3 a A | 3.1 a AB |
| | Flesh | 23.1 a A | 2.0 a B | 7.5 a B | 209.5 b C | 36.5 b C | 2.6 a AB |
| | Seed | 0.0[y] b C | 0.6 b C | 0.8 b C | 189.3 b C | 39.2 b BC | 0.1 b B |
| | P value | <0.0001 | 0.0008 | <0.0001 | <0.0001 | <0.0001 | <0.0001 |
| 2013 | Whole | 15.7 a B | 3.1 a A | 10.7 a A | 298.8 a B | 58.5 a A | 5.7 a A |
| | Flesh | 10.7 b B | 2.8 a AB | 6.9 b B | 155.4 b CD | 47.8 b B | 3.9 ab A |
| | Seed | 0.0 c C | 0.4 b C | 0.8 c C | 124.1 b D | 27.6 c D | 0.1 b B |
| | P value | <0.0001 | 0.0004 | <0.0001 | <0.0001 | <0.0001 | <0.0140 |

[z] Means followed by the same letter are not statistically different ($p<0.05$) by year (lowercase) and across years (uppercase), separated with Tukey's HSD.
[y] 0.0 = concentrations lower than detectable level using HPLC.
[x] ORAC = Oxygen Radical Absorbance Capacity (µmol Trolox Equivalents/g).

*Total Phenolics.* Overall total phenolics in whole berries and seeds were greater in 2012 compared to 2013 (Table 1). Total phenolics in the seeds and whole berries of 'Supreme' were lower than those reported by Striegler et al. (2005). Conversely, Pastrana-Bonilla et al. (2003) reported lower total phenolics in the whole berries, and higher total phenolic concentrations in the seeds in 'Supreme' than found in our study. Threlfall et al. (2005) found total phenolic concentrations in the seeds of 'Black Beauty' to be greater than reported in our study. Total phenolics were highest in the whole berries (Table 1), which was contrary to the findings of Sandhu and Gu (2010), who found total phenolics to be highest in seeds. For total phenolic content, 53% was found in flesh while 47% was in the seed in 2012, and 56% was in the flesh and 44% was in the seed in 2013.

*ORAC.* ORAC levels in whole berries of 'Supreme' were higher while the ORAC levels found in the seeds were lower than those reported by Striegler et al. (2005). Threlfall et al. (2005) found ORAC levels in the seeds of the black muscadine cultivar 'Black Beauty' that were higher than our findings (Table 1). Interestingly, ORAC levels of seeds were greater in 2012 than in 2013, while the flesh had higher ORAC levels in 2013 compared to 2012 (Table 1). In 2012, 48% of ORAC was accounted for in flesh and 52% in seed, conversely in 2013, 63% was accounted for in flesh and 37% in seed. ORAC was positively correlated with total phenolics ($r = 0.88$), showing that phenolic compounds are the major contributor to antioxidant capacity, which agreed with the findings of Pastrana-Bonilla et al. (2003).

*Resveratrol.* Resveratrol concentrations reported in muscadine skins (Magee et al., 2002) and whole berries (Ector et al., 1996) were similar to that of this study. The levels of resveratrol in the seeds, flesh and whole berries among years did not change (Table 1). This was surprising as the wetter and cooler growing season of 2013, could likely result in higher disease incidence and therefore increasing resveratrol production by the berries in response to pathogen infection**.** For resveratrol content, 96% was found in the flesh and 4% in the seed for both years of the study.

*Changes in nutraceutical compounds during storage.*

The *F*-test indicated significant interactions for the nutraceutical compounds between storage period and berry segments for total anthocyanins ($P = 0.0226$), total ellagitannins ($P = 0.0018$), ORAC ($P < 0.0001$),

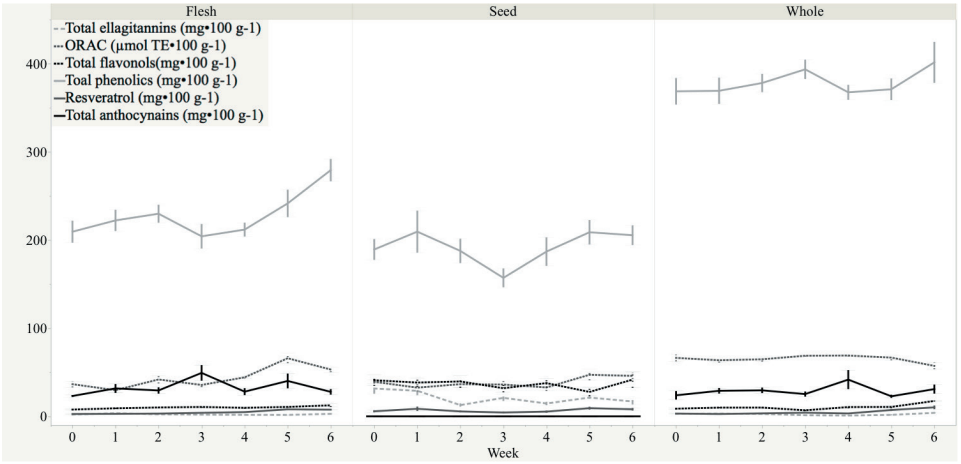


**Fig. 1.** Retention of nutraceutical compounds of 'Supreme' muscadine (*Vitis rotundifolia* Michx.) whole berry and berry segments during storage at 2°C and 85-95% RH for 6 weeks (2012). ORAC = Oxygen Radical Absorbance Capacity (µmol Trolox Equivalents·g$^{-1}$). SE bars were constructed using one SEM.

total flavonols ($P < 0.0001$), and resveratrol ($P < 0.0001$). However, the interaction differences were small and most compound differences remained relatively constant during storage (Fig. 1). Marshall and Stringer (2014) also found that total anthocyanins, total phenolics, and ORAC generally did not change during storage for two weeks. Lee and Talcott (2002) found that total ellagitannins in muscadine juice and wine remained relatively stable during storage for 50 d, which is similar to our findings. Contrary to the findings of our study, Marshall and Stringer (2014) found that total ellagitannins generally increased during storage for two weeks although they found variation among genotypes. Storage retention of total flavonols has not been previously studied in muscadines. Kalt et al. (1999) suggested that storage at ambient or above ambient temperature can positively affect phenolic metabolism and enhance antioxidant capacity of small fruit. However, maintenance of similar levels of total phenolics during storage found in our study might be attributed to storage at 2°C, a temperature that likely was low enough to suppress metabolic activity. Similar to our study, Marshall and Stringer (2014) found that during storage, resveratrol did not significantly change in six cultivars, but they did find resveratrol increased in five and decreased in three cultivars.

## Conclusions

Both the findings that postharvest storage of muscadine grapes did not dramatically impact nutraceutical components in the whole berries or segments along with the identification and quantification of nutraceutical components in our study provided more insight into nutraceutical aspects of this much underutilized fruit. Further, the differences among years for certain variables indicated the importance of multi-year evaluations for certain nutraceutical compound levels. This could be of particular value if new cultivars are released with nutraceutical levels reported and these values should be measured from multiple years of observation.

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