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Ellagic Acid and Ellagitannins Affect on Sedimentation in Muscadine Juice and Wine

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A mechanism for the formation of water-insoluble sediments in wines and juices made from red and white muscadine grapes (*Vitis rotundifolia*) was investigated as a function of processing methodology and storage. Sediments are considered quality defects in muscadine grape products, and their presence may influence consumer acceptability and expansion of retail markets. Processing regimes included both hot (70 °C) and cold (25 °C) press techniques for wine or juice production, and fermentations in contact with grape skins for 3, 5, and 7 days. Relationships between free ellagic acid (FE), total ellagitannins (ET), and total ellagic acid (TE) concentrations were evaluated initially in each product and in sediments that formed during storage for 50 and 120 days at 20 °C. Processing techniques influenced initial concentrations of these compounds and the extent of sediment formation. Following storage, juices generally had higher concentrations of FE in sediments compared to wines, but sedimentation was independent of initial FE or TE concentrations. Decreases in ET were observed for hot-pressed juice and skin-fermented wines after storage indicating their hydrolysis during storage and possible contribution to FE in sediments. However, quantitative analysis of the collected sediments revealed that no more than 12% FE by weight was actually present in the sediments, with the remainder consisting of either unidentified compounds or conjugated forms of ellagic acid. This work elucidated a potential mechanism for the presence of FE in muscadine wine and juice sediments through ellagitannin hydrolysis and suggests that sedimentation from mechanisms other than ellagic acid precipitation may also contribute to wine and juice quality.

KEYWORDS: Muscadine; ellagic acid; ellagitannin; sediment; wine; juice

INTRODUCTION

Muscadine grapes (*Vitis rotundifolia*) are a valued fruit crop in the southeastern U.S. because other grape varieties are difficult to cultivate in the humid summers and warm winters characteristic of these regions. Muscadine grapes are commonly used for wine and juice production, but suffer from several quality defects including poor color stability and the formation of insoluble sediments during storage. The latter is considered a visual deterrent to consumers and may discourage future purchase of muscadine grape products. Previous reports have identified ellagic acid as the predominant component of these sediments (*1*, *2*), but no effective treatments or processing unit operations have proven successful in preventing ellagic acid precipitation on an industrial scale.

Ellagic acid is a dimeric derivative of gallic acid and is generally recognized as the hydrolytic byproduct following the release of a hexahydroxydiphenoyl (HHDP) ester group from ellagitannins (**Figure 1**), which spontaneously converts to its characteristic bislactone structure (*3*). Ellagic acid is present in many woody plants, fruits, and nuts, and over 500 different ellagitannins have been identified in nature (*4*). Ellagic acid and ellagitannins have important biological functions and can scavenge both superoxide and peroxyl radicals in solution (*5*). Ellagitannins are common in wines aged in oak barrels, because the wood of some varieties may contain up to 10% ellagitannins by weight (*6*), contributing to the sensory properties of a wine (*5*, *7*).

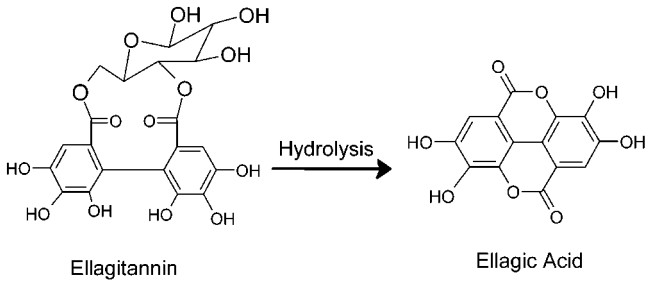

Figure 1. Conversion of ellagitannin into ellagic acid via hydrolytic release of hexahydroxydiphenoyl (HHDP).

Remediation of ellagic acid sedimentation in muscadine wine and juice has not been successfully accomplished, owing to the low solubility of free ellagic acid (FE) in solution and the undetermined role of ellagitannins. Thus far, ellagic acid has

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been the only identifiable compound in the sediments, and it may form by slow precipitation during storage leaving a yellow or red flocculent at the bottom of storage vessels (*1*, *2*, *8*, *9*). The extent of sedimentation was greater with longer skin-fermentation times (*9*) and was accelerated following pasteurization or storage at elevated temperatures (*8*). The source of ellagic acid in sediments may result from precipitation of FE or by hydrolysis of larger ellagitannins as hypothesized by Musingo (*10*). However, no studies have confirmed hydrolytic breakdown of ellagitannins during storage and its relationship to the formation of sediments in muscadine grape products.

The objectives of this study were to determine the relationships between FE, total ellagitannins (ET, sum of individual ellagitannins), and total ellagic acid (TE, sum of FE and ellagic acid derived from ellagitannins after acid hydrolysis) in muscadine wine and juice prepared by various processing techniques. Analysis of these compounds before and after storage for 50 and 120 days at 20 ± 1 °C helped determine causative factors leading to the formation of water-insoluble sediments during storage. This work identifies precursors to the formation of ellagic acid sediments in muscadine wine and juice that may lead to industrial processes for remediation of this quality defect.

MATERIALS AND METHODS

Juice and Wine Preparation. Muscadine juice is normally manufactured by one of two extraction techniques depending on the desired color and sensory characteristics of the final product. Typical juices may be a blend of hot-pressed (HP) and cold-pressed (CP) juices, as the former tends to be very astringent and the latter has lower acid and pigment content (*11*). Muscadine wines are made in a manner similar to those from *Vitis vinifera*, with fermentations usually following crushing and skin contact for several days. Muscadine grapes have characteristic thick skins making extraction of polyphenolic compounds difficult without the aid of heat, macerating enzymes, or ethanol; a typical grape will yield 50−65% juice by weight for CP and 60−75% juice for HP techniques (*12*).

For this study, two muscadine grape varieties (red and white) were obtained from a local grower in central Florida and processed into wine and juice by different extraction techniques. The white cultivar (Carlos) was crushed and pressed in a hydraulic basket press (Prospero's Equipment, Cort, NY) with 25 mg/L potassium metabisulfite ($K_2S_2O_5$) added to retard oxidation. This process was referred to as "cold-press" technique, but grapes were pressed the day of harvest at an extraction temperature of ∼25 °C. A portion of this juice was frozen and stored at −20 °C until needed, and the remaining juice was adjusted to 20% soluble solids with sucrose, inoculated with yeast (Premier Cuvee, Universal Foods Corporation, Milwaukee, WI), and fermented to dryness (<0.1% reducing sugar) at 13 °C. The wine was then racked under nitrogen, additional sulfites were added (25 mg/L), and the wine was cold stabilized (4 °C for 4 weeks) to precipitate tartaric acid. Wine and juice were obtained in a similar manner for the red cultivar (Noble), but 50 mg/L sulfite was added to retard oxidation.

HP juice was obtained by heating crushed Noble grapes for 15 min at 70 °C prior to pressing, and varying the times of skin-contact during initial stages of fermentation produced three additional wines. For these wines, grapes were crushed, inoculated with yeast, and held at 20 °C for 3, 5, and 7 days prior to pressing the partially fermented must. Soluble solids were then adjusted to 20% with sucrose and the fermentation was completed as previously described. All juice samples were kept frozen (−20 °C) until fermentations were complete, and were analyzed simultaneously with wines in order to minimize variability. Finished wines and juices were then filtered through cellulose filter pad filters (Cellulo Co., Fresno, CA) with a 2-cm bed of diatomaceous earth and bottled; juices were subsequently pasteurized to an internal temperature of 90 °C for 1 min.

These processing methodologies resulted in eight muscadine products for analysis that included Carlos CP wine and juice, Noble CP wine and juice, Noble HP juice, and Noble wine pressed after 3, 5, and 7 days of skin contact time. Research samples of wine and juice were treated with sodium azide (50 mg/L, Sigma Chemical Co., St. Louis, MO) to prevent microbial growth during storage.

Phytochemical Assessments. HPLC analysis of FE and ET were performed using a Waters Alliance 2690 system equipped with a Nova-Pak C_{18} column (150 mm × 3.9 mm, Waters, Milford, MA) and a Waters 996 photodiode array detector recorded between 200 and 400 nm; ellagic acid was quantified at 360 nm. Aliquots of wine and juice were filtered (0.45 μm) prior to injection and analyzed without further preparation for FE and ET. Identical aliquots were also analyzed for TE following acid hydrolysis for 60 min at 100 °C in 2 N HCl adjusted to 50% methanol. HPLC mobile phase consisted of water (phase A) and 60% methanol (phase B) both adjusted to pH 2.4 with *o*-phosphoric acid and ran at 1 mL/min. A gradient elution program ran phase B from 0 to 30% in 3 min, 30−50% in 2 min, 50−70% in 5 min, 70−80% in 5 min, and 80−100% A in 2 min for a total run time of 17 min after which the column was equilibrated to original conditions for the next sample injection. Calibration curves ($R^2 = 0.99$) for ellagic acid (Sigma) dissolved in 70% methanol were used to quantify FE and ET.

Separation and identification of FE in muscadine wine and juice were assessed compared to the authentic standard of ellagic acid and ET confirmed from ethyl acetate extracts of Noble grapes. These isolates were obtained by extracting juices with two vol of ethyl acetate to partition FE, individual ellagitannins, and other phenolic compounds from anthocyanins and other interfering compounds (*13*). Ethyl acetate extracts were pooled, evaporated to dryness, and dissolved in water at pH 3.1 for HPLC analysis. FE and individual ellagitannins were identified based on retention time and UV spectral properties before and after acid hydrolysis. All solvents used were of HPLC grade and were purchased from Fisher Scientific (Fair Lawn, NJ).

FE in wine and juice sediments were assayed before and after storage for 50 days at 20 °C by filtering a 10-mL aliquot through a 0.45-μm syringe filter and washing with water to remove soluble material. Insoluble residues were then eluted with 10 mL of 70% methanol for HPLC analysis. Subsequently, FE was also quantified in sediments of the skin-fermented wines after 120 days storage by filtering 50 mL through a preweighed 0.45-μm membrane filter using a vacuum manifold. Collected sediments were washed with water, dried in a desiccator, weighed, and dissolved in 100% methanol for 12 h for determination of FE concentration by HPLC. On the notion from previous investigators that these sediments were primarily composed of ellagic acid, a theoretical concentration (w/v) of ellagic acid was determined by which comparisons were made to actual concentrations.

Statistical Analysis. Determination of Pearson correlation coefficients, regression models, and analyses of variance were conducted using SAS, Version 6.12 (SAS Institute, Inc, Cary, NC). Mean separation was performed using Duncan's multiple-range test ($P < 0.05$). The experiment was randomized and conducted in triplicate except where noted.

RESULTS AND DISCUSSION

Identification and Confirmation of FE and Ellagitannins. FE and ET in muscadine juice and wine were identified by HPLC based on retention time and UV spectral properties as compared to an authentic standard of ellagic acid (**Figure 2**). Spectral properties of ellagic acid indicated absorbance maximums at 253.6 and 366.2 nm (**Figure 3**) as confirmed by refs *14* and *15*. The spectral band at 253.6 nm is represented by two benzene rings in the structure, and a second band at 366.2 nm is the result of two oxo-groups adjacent to hydrolyzable lactones that are altered spectrally during alkaline hydrolysis (*16*, *17*). Three ellagitannins were identified, each sharing identical spectral properties, and because authentic standards are not commercially available, were summed together for data analysis. Similar to ellagic acid, ellagitannins had an absorption maximum at 253.6 nm but were slightly different in the second spectral band, most likely due to the presence of either a sugar

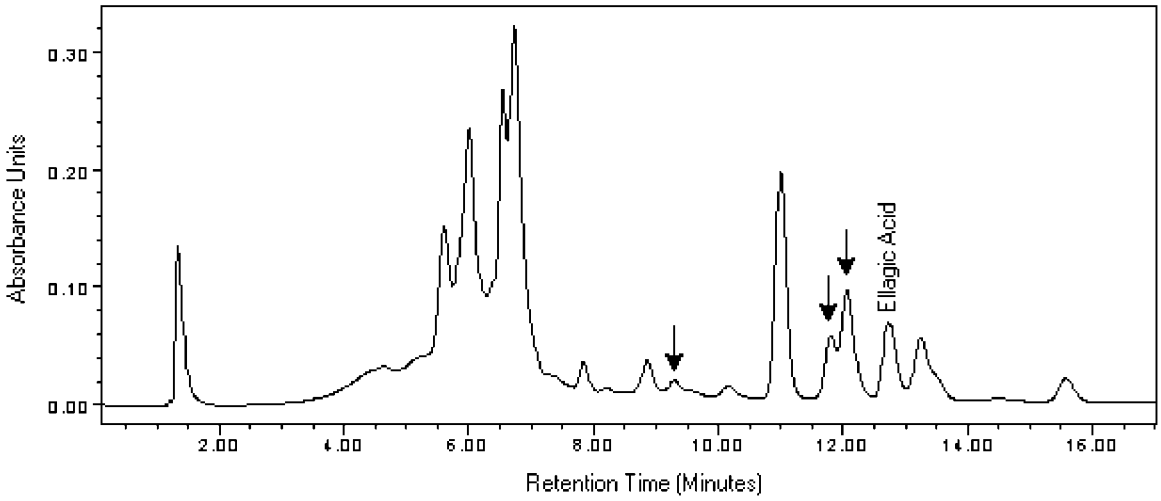

Figure 2. Reversed-phase HPLC chromatograph of Noble hot-pressed juice prior to storage or acid hydrolysis. Free ellagic acid and three ellagitannins (solid arrows) were separated from anthocyanin and flavonol glycosides (other chromatographic peaks).

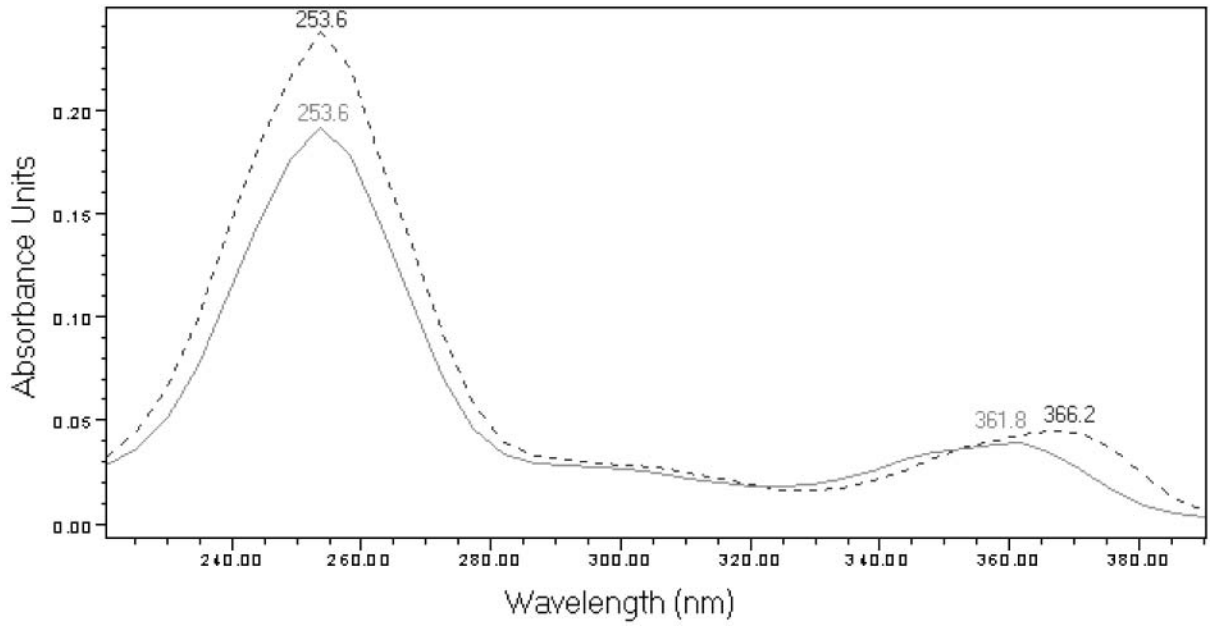

Figure 3. UV spectrum of free ellagic acid (dotted line) and an ellagitannin (solid line) identified in hot-pressed muscadine juice (see **Figure 1**). Spectral properties of free ellagic acid were identical to those of an authentic standard, and ellagitannins were identified on the basis of similar spectral characteristics. Spectral differences were likely the result of hexahydroxydiphenoyl units on the ellagitannin and the lack of a bislactone structure.

moiety or HHDP units instead of the typical lactone in ellagic acid. Three criteria were used to confirm these compounds as ellagitannins, including UV spectra, polarity (ellagitannins should elute prior to ellagic acid on a reversed-phase column), and the disappearance of ellagitannins upon acid hydrolysis with a corresponding increase in FE. Ethyl acetate extracts were instrumental in confirming the presence of ellagitannins by removing interfering compounds.

FE and ET in Muscadine Wine and Juice. Processing methodology and cultivar influenced the initial concentrations of FE and ET in muscadine grape wine and juice (**Table 1**, $P < 0.05$). From identical juice presses, muscadine juices contained higher concentrations of FE and ET compared to those in the wines. FE in Carlos CP juice was nearly twice the concentration found in wine and contained ellagitannins that were not detectable in the wine. A similar trend was observed for Noble CP juice, but neither FE nor ET was detected in the corresponding wine. However, subsequent analysis of this wine following binding and partitioning and from a Waters C_{18} Sep Pak showed trace levels of FE (data not shown) that were apparently masked by interfering compounds. The observed disparity in concentrations between wine and juice samples were attributed to losses during wine production, which were greater than losses associated with juice pasteurization. Concentrations of FE and ET were appreciably higher in Noble HP juice compared to CP juice, demonstrating the role of heat extraction in releasing polyphenolics from grape skins. However, skin-fermented wines were comparable to Noble HP juice for extraction of pigments and based on visual appearance. Comparing Noble HP juice to skin-fermented wines was vital to determining conditions favorable for sediment reduction. Concentrations of FE were higher in skin-fermented wines than in HP juice, but ET and TE levels were higher in HP juice, indicating possible hydrolysis of ellagitannins during the wine-making process. The 3- and 5-day skin contact times extracted the highest concentrations of FE and ET, but levels were lower in the 7-day skin contact wines, apparently due to oxidation or sedimentation.

Table 1. Concentrations (mg/L) of Free Ellagic Acid, Total Ellagitannins, Total Ellagic Acid, and Free Ellagic Acid in the Sediments of Carlos and Noble Wines and Juices before and after Storage at 20 °C for 50 Days[a]

cultivar	processing	treatment[c]	free ellagic acid		total ellagitannins[b]		total ellagic acid		free ellagic acid in sediment	
			initial	day 50	initial	day 50	initial	day 50	initial	day 50
Carlos	juice	CP	3.14 e	3.08d	0.83 d	0.76 e	20.51 d	15.91 d*	ND[d]	0.75 b
Carlos	wine	CP	1.62 f	1.89 d	NDe	ND e	13.19 e	12.09e	ND	0.30d
Noble	juice	CP	3.85 d	2.55 e*	0.64 de	0.42 e	8.44 f	7.85 f	ND	0.68 bc
Noble	wine	CP	ND g	ND e	ND e	ND e	2.27 g	2.63 g	ND	0.33 cd
Noble	juice	HP	10.14 c	9.72 c	22.66 a	19.05 a*	84.65 a	43.22 c*	ND	2.42 a
Noble	wine	3 day	16.19 a	19.81 a*	13.48 b	5.83 b*	59.60 c	48.91 b*	ND	0.62 bcd
Noble	wine	5 day	16.23 a	19.40 a*	13.27 b	4.85 c*	64.49 b	52.34 a*	ND	0.70 b
Noble	wine	7 day	12.73 b	16.29 b*	11.48 c	3.09 d*	65.30b	51.67 ab*	ND	0.57 bcd

[a] Processing methodology is described in Materials and Methods. Similar letters within columns indicate that processing treatments were not significantly different (Duncan's test, $P < 0.05$, $n = 3$). Asterisk (*) indicates significant difference between initial levels and 50 days of storage. [b] Expressed as ellagic acid equivalents. [c] Treatment abbreviations. CP, cold-pressed. HP, hot-pressed. Skin contact times (3, 5, and 7 days). [d] ND, concentrations below detection limit.

TE was quantified following acid hydrolysis, which released appreciable amounts of FE into solution. A 1:1 conversion of ellagitannins to ellagic acid was not expected, because multiple HHDP units can be esterified to a simple sugar such as glucose. A regression model was obtained (ET $= -2.28 + 0.32 \times$ (TE $-$ FE)) to predict ET in muscadine wine and juice ($R^2 = 0.94$) by subtracting FE from TE to better represent hydrolyzable ellagitannins. Despite the high regression coefficient, additional ellagitannins were likely present that were not detected by our chromatographic conditions. For example, ellagitannins were not detected in Carlos and Noble CP wines but FE was readily detectable following acid hydrolysis. Quantification of ellagitannins in muscadine wine and juice and evaluation of their potential to release ellagic acid into solution are important for assessing factors contributing to ellagic acid sedimentation.

Storage Effects on FE and ET in Muscadine Wine and Juice. Muscadine grape products are prone to sediment development during storage, and ellagic acid was previously identified as the principal component in these sediments. To further investigate mechanistic causes for sedimentation in wines and juices, the wines and juices were stored for 50 days at 20 °C, and changes in FE and ET monitored. The relatively short storage period was sufficient for observing significant changes in these compounds as influenced by the various processing parameters (**Table 1**, $P < 0.05$), and the greatest changes during storage were observed for the polyphenolic-rich Noble muscadine cultivar. FE was unchanged or decreased for all juice samples during storage, whereas FE in wines increased from 9 to 28%. The increase in FE observed for wine was paralleled by a corresponding decrease in ET ($r = -0.82$), decreasing 63−79% in skin-fermented wines compared to a 16% loss in Noble HP juice. Consequently, decreases in TE were greater (49%) for HP juice compared to only 18−21% for skin-fermented wines. Various possibilities exist for the changes observed during storage including oxidative loss, hydrolytic breakdown of ellagitannins, and the formation of insoluble sediments. First, visual browning from autoxidative reactions was noticeable in all samples and therefore considered as a plausible factor. Next, the short and relatively mild storage conditions employed in this study were not expected to result in complete hydrolysis of all ellagitannins, but appeared to be instrumental in increasing FE. Finally, visual sediments formed in all wines and juices after 50 days of storage were collected for analysis of FE. Levels of FE in sediments were very low compared to levels present in solution, but concentrations may reflect low solubility characteristics during recovery/solubility procedures from a 0.45-μm syringe filter. The ethanol content of wines may also have influenced solubility characteristics, as saturation limits in ethanol are higher than those of water (*18*), contributing to greater sediment formation in juices. Noble HP juice had the highest concentration of FE in its sediment, whereas concentrations in Noble and Carlos CP juices were both equivalent to those in skin-fermented wines, indicating a poor relationship between FE in sediments and processing conditions affecting initial polyphenolic concentrations.

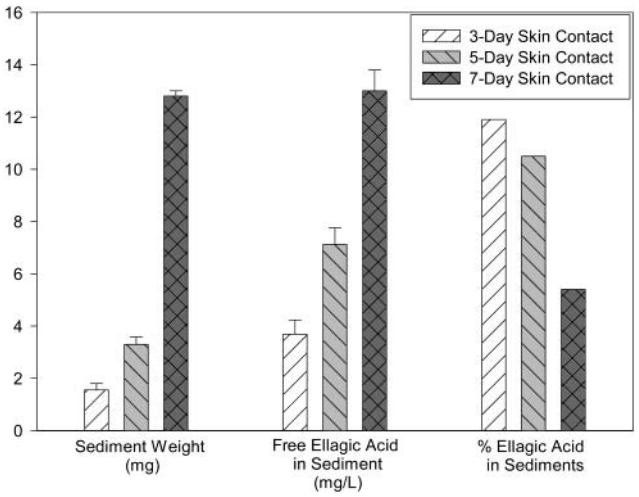

Figure 4. Mass balance of sediments collected from 3-, 5-, and 7-day skin-fermented wines after 120 days of storage at 20 °C. Bars indicate the actual sediment weight (mg), concentration of free ellagic acid (mg/L), and percent free ellagic acid in sediments calculated as if the sediments were composed of only ellagic acid. Bars indicate standard error of the mean ($n = 2$).

Mass Balance of Muscadine Sediment. In an effort to further elucidate the contribution of ellagic acid to sediments formed in muscadine products, FE was quantitatively determined in sediments from skin-fermented wines after 120 days of storage at 20 °C (**Figure 4**). Previous work elucidating FE in sediments emphasized that sediments were almost exclusively composed of ellagic acid, with little consideration given to the presence of additional compounds. In subjective observations,appreciable amounts of sediment appeared in each sample after storage that could not be solely attributed to ellagic acid based on FE concentrations. Therefore, we hypothesized that if sediments were composed primarily of FE then actual concentrations quantified by HPLC would be equivalent to the calculated or theoretical concentration based on sediment weight per volume. Sediments were collected on preweighed 0.45-μm membrane filters and solubilized in 100% methanol for 12 h. Sediment weights increased with time of skin contact during

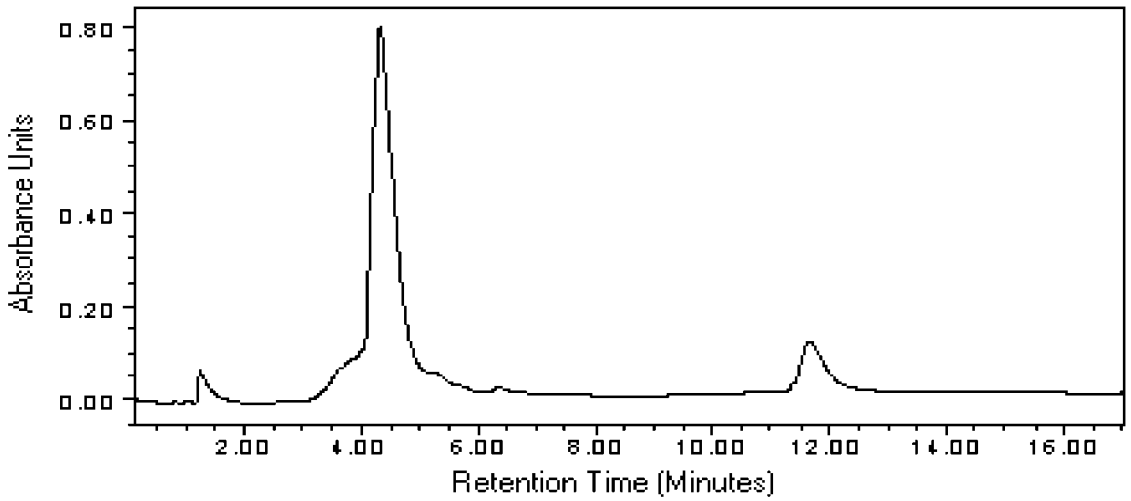

Figure 5. HPLC chromatograph obtained after solubilization of sediments from 7-day skin-contact muscadine wine detected at 220 nm. Free ellagic acid (t_r = 11.8 min) was readily detectable at this wavelength, but an additional compound with strong UV absorption characteristics could not be conclusively identified (t_r = 4.4 min).

fermentation, reaching a maximum of 12.8 mg in a 50-mL aliquot in 7-day skin contact wines. Levels of FE were still low compared to those of TE, but were higher than those found after 50 days of storage. The calculated concentration of FE based on sediment weight was found to be considerably lower than actual concentrations, with >88% of the sediment being composed of compounds other than FE. Extraction of compounds contributing to sediment formation was greatly enhanced with increased skin contact time as reflected in the inverse relationship with %FE in the sediments. Visually, collected sediments had appreciable amounts of insoluble residues even after methanolic extraction for 12 h, and no significant increase in FE was observed after acid hydrolysis, demonstrating their poor solubility characteristics. Two additional compounds in the sediment were also detected by HPLC: an ellagitannin present in trace amounts and an unknown compound with an absorption maximum at 220 nm (**Figure 5**). The incomplete quantification of FE and significant losses of TE during the storage of wine and juices may also indicate the presence of ellagic acid conjugates that could not be solubilized or detected by the methodologies employed in this study. Other compounds in the sediments may consist of polymerized anthocyanins due to the sediment color, tannin−protein polymers, or insoluble pectic substances, all of which may be influenced by wine and juice processing methodologies.

CONCLUSIONS

Changes in FE and ET present in muscadine wines and juices were evaluated initially and after 50 days of room temperature storage. Processing methodology had a significant impact on the concentrations of these compounds, and hydrolysis of ellagitannins was believed to be instrumental in contributing to FE in sediments. Overall, juices contained higher concentrations of FE in their sediments compared to wines, a likely consequence of ethanol content of wines and changes during fermentation. Despite an inverse correlation between FE and ET, the sediments were found to contain low levels of FE with the remainder consisting of unidentified compounds. Further elucidation of the compounds contributing to sediment formation, and development of industrial steps for remediation, is important for improving the quality of muscadine wine and juice.

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