Food Sci. Biotechnol. 25(5): 1319-1326 (2016)
DOI 10.1007/s10068-016-0207-3

Food Science
and
Biotechnology

Characterization of volatile compounds in Cowart muscadine grape (*Vitis rotundifolia*) during ripening stages using GC-MS combined with principal component analysis

Bolim Lee[1], Pei-ching Lin[1], Hwan soo Cha[2], Jun Luo[3], and Feng Chen[1,4,*]

[1]*Department of Food, Nutrition and Packaging Sciences, Clemson University, Clemson, SC, 29634, USA*
[2]*Korea Food Research Institute, Seongnam, Gyeonggi 13539, Korea*
[3]*Department of Mathematical Sciences, Clemson University, Clemson, SC, 29634, USA*
[4]*Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Technology and Business University, Beijing 100048, China*

Received May 20, 2016
Revised July 4, 2016
Accepted July 6, 2016
Published online October 31, 2016

*Corresponding Author
Tel: +864-656-5702
Fax: +864-656-0331
E-mail: fchen@clemson.edu

pISSN 1226-7708
eISSN 2092-6456

Abstract Muscadine grape (*Vitis rotundifolia*) is a popular fruit in the Southeastern United States because of its unique aroma and strong antioxidant capacity. Volatile compounds of a locally cultivated muscadine cultivar Cowart were characterized by solid-phase microextraction coupled with GC-MS. Twenty-eight volatile compounds, including fruity short-chain esters, alcohols, terpenes, and carbonyl compounds, were detected based on mass spectra and Kovats indices. Based on principal component analysis and hierarchical clustering, the grapes in stages I and II had relatively similar flavor patterns, which were different from that in stage III. Butyl-2-butenoate, hexyl acetate, propyl acetate, ethyl trans-2-butenoate, hexyl-2-butenoate, ethyl acetate, butyl acetate, 1-octanol, ethyl hexanoate, and β-citral were present as distinct volatile chemicals in stage III, while nonanal, decanal, and β-citronellol were distinct in stage II, and myrcenol, β-ocimene, and l-limonene were biomarkers in stage I. Understanding volatile compounds at each stage can assist farmers in choosing the optimal time to harvest muscadine grapes.

Keywords: muscadine, aroma, gas chromatography-mass spectrometry, principal component analysis, solid phase microextraction

Introduction

Muscadine grapes (*Vitis rotundifolia*) were a native American grape species cultivated in the Southeastern United States. They have adapted to a warm–humid climate and well-drained soil. They grow well over a vast area, from Delaware to Central Florida, the Atlantic Ocean to East Texas, and along the Mississippi River to Missouri (1). For decades, their inherent bioactive phytochemicals have drawn increasing attention owing to their high antioxidant capacity compared to those of other grapes and fruits, as well as their rich content of phytochemicals that contribute significantly to the prevention of human diseases (2).

Volatile compounds in table grapes, grape juice, and wines have been studied for a long time because aroma is a major factor for consumer acceptance. Those aromas comprise hundreds of volatile organic compounds (VOCs) made up of different chemical groups, including alcohols, esters, aldehydes, ketones, monoterpenoids, and C_{13} norisoprenoids. However, the concentration of each VOC varies significantly depending on many factors, such as cultivars, abiotic and biotic stress factors, and ripening stages (3-6). In addition, an accurate determination of unstable VOCs in trace amounts is challenging and often involves the consideration of many factors, such as the solvent's polarity, extraction method, and instrument sensitivity. Most of the VOCs in grapes are free VOCs that directly contribute to aromas, while the bound glycosidic forms are odorless and considered to be the flavor precursors (7,8).

Despite much research on the flavor chemistry of grapes, information on the VOCs of muscadine grapes (*Vitis rotundifolia*) in their ripening stages is limited. In fact, the production of one muscadine cultivar Cowart has decreased in recent years despite its relatively desirable flavor and taste. Therefore, in order to promote the cultivation of Cowart, characterization of its VOCs during its different ripening stages along with the fruit maturity is important. In general, there are three main stages of berry development, i.e., stage I (green), stage II (pink), and stage III (black; fully mature) (9). Stage I includes the period of rapid cell division, but no softening occurs in this stage. In stage II, the berries change colors from green to pink, and cell expansion begins. Stage III results in the purple and black coloring of mature grapes having maximum accumulation of anthocyanins, and presents a softened texture (10).

During the ripening stages, free and glycosylated VOCs are accumulated (11). The physiological changes of grapes during the ripening stages include not only physical changes such as weight, color, and fruit rigidities, but also chemical changes such as pH, contents of sugar, alcohol, phenolics, and the acidity level (12,13). Thus, it is important to understand the compositional and aromatic changes that occur in fruits during the ripening stages to evaluate the desirable quality and develop a connection between the grape quality and its aromas, which provides a predictive marker that can assist in determining the optimal harvest time.

Methods for extracting VOCs often include liquid–liquid extraction, simultaneous distillation and extraction, headspace solid-phase microextraction (HS-SPME), and stir bar sportive extraction techniques (14). However, the use of solvent extraction methods has some disadvantages, such as possible sample contamination, an environmental burden, and loss of the VOCs owing to their degradation during the concentration step (4). In comparison, SPME is considered a more desirable technique for many flavor analyses because it is a rapid, inexpensive, and solvent-free technique that combines the extraction and concentration steps in the headspace (15). Thus, SPME has been widely used in many liquid and fragrance analyses. The aims of this research were to: (1) identify the VOCs extracted from muscadine grapes in the three developmental stages using the HS-SPME technique; and (2) compare the volatile profiles of muscadine grapes using a principal component analysis (PCA).

Materials and Methods

Materials, chemicals, and reagents A manual SPME holder and mixed coating fiber (DVB/CAR/PDMS, 50/30 μm) were purchased from Supleco (St. Louis, MO, USA). Glass tubes (40 mL) and poly-tetrafluoroethylene (PTFE)/silicone septa were purchased from Scientific Specialities Service (Hanover, MD, USA). High-performance-liquid-chromatography grade dichloromethane (DCM), water, and sodium chloride (NaCl) were obtained from Fisher Scientific (Hampton, NH, USA). Alkane standard chemicals (C_8–C_{20}) and an internal standard (6-methyl-5-hepten-2-ol, 99.9% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation One cultivar of the black-skinned muscadine grape, Cowart, was randomly harvested from a local farm, Happyberries (Seneca, SC, USA) over six weeks (July 24–September 5, 2014) during its growing season. The Cowart berries were picked up in three stages. The stage I muscadine grapes were harvested on July 24, 2014 when the grapes were small green fruits. The stage II grapes were harvested on August 22, 2014 when the grape fruits became soft and translucent and their skin color turned from green to pink/red. The stage III grapes were harvested on September 5, 2014 when its skin color turned from purple to black when harvested. All the samples were assessed based on subjective evaluation of color changes as their skin colors were obviously different in the three ripening stages. After the harvest, all the samples were freshly extracted for volatile chemical analyses or packed under vacuum and stored at 20°C for nonvolatile chemical analyses.

Optimization of solid-phase microextraction (SPME) method First, optimization of the SPME method for volatile extraction from the muscadine grapes was performed based on previously reported methods (3). Three hundred grams of fresh samples from each of stages I, II, and III were blended using a commercial blender and transferred into 50-mL plastic centrifuge tubes, and then centrifuged at 1,610× *g* for 30 min at 5°C. The supernatant was transferred to five glass tubes (40 mL) and capped by PTFE/silicone septa. Then, samples were separated into two parts: (a) 20 mL of each sample supernatant were mixed with NaCl at a final concentration 0.2 g/mL of NaCl after moving into a tube (40 mL), and (b) 20 mL of each sample supernatant as a control were not added with NaCl. In addition, the internal standard (6-methyl-5-hepten-2-ol) in a concentration of 4,000 ppm was added in both samples. The SPME method was optimized based on three parameters (i.e., extraction time, extraction temperature, and ion strength of the sample solution). The extraction times were selected as 15, 30, 60, and 90 min, the extraction temperature as 40, 50, 60, and 70°C, and the ion strength of the extraction solution was tested by the blank and unsaturated salt solution (0.2 g/mL of NaCl) in order to obtain the best conditions for extraction of muscadine volatile compounds. The effects of extraction time, temperature, and ion strength on the chromatographic responses of three representative volatile compounds, i.e., 2-hexenal, 1-octanol, and *trans*-geraniol, were used for the extraction optimization. After preliminary tests, the optimal experimental condition for extracting the VOC of muscadine grapes was set at 60°C with 30 min of extraction time for the solution of grape supernatant with 0.2 g/mL of NaCl.

Moreover, each sample was placed in a water bath for 30 min for headspace equilibrium before the fiber was inserted into the bottle for volatile sampling. After the extraction, the fiber was transferred to the injection port of the GC to desorb the volatile compounds for 3 min. The SPME fiber was conditioned at 250°C for 5 min in the GC injector, based on the manufacturer's recommendation. All extraction samples were repeated in triplicate.

GC-MS analysis A Shimadzu GC-17A gas chromatograph (GC), which was coupled to a QP5050A mass spectrometer (Shimadzu, Kyoto, Japan) and equipped with an Agilent Technologies (Santa Clara, CA, USA) DB-5 (5% Phenyl, 95% methyl silicone) (60 m length× 0.25 mm ID×0.25 μm film thickness) capillary column, was used to analyze volatile compounds. The volatile compounds were extracted by a SPME fiber (DVB/CAR/PDMS) (Restek Corporation, Bellefonte, PA, USA) in size of 0.77 mm ID and manually injected into the GC injector port for 3 min to thermally desorb the volatile compounds in a splitless mode. The ultra-high purity (UHP) helium gas was adopted as the carrier gas in a flow rate at 1.0 mL/min. The initial oven

(A) Stage I (B) Stage II (C) Stage III

Fig. 1. Three ripening stages of a muscadine grape (*Vitis rotundifolia*) cultivar, Cowart

temperature was set at 40°C and held for 5 min, then increased from 40 to 100°C at 2.5°C/min and held for another 1 min, and then increased from 100 to 150°C at 3.0°C/min, and finally increased from 150 to 265°C at 20°C/min and held for 5 min. The temperatures of the GC injector and interface between GC-MS were set at 250 and 250°C, respectively. The mass spectrometer was operated in an electron ionization (EI) mode at 70 eV, and chromatogram was recorded in a SCAN mode in a mass range of 40–350 *m/z* from 1.5 to 57 min.

Volatile chemical identification Identification of volatile compounds was based on matching their mass spectra with the NIST 08 library (National Institute of Standards and Technology, Gaithersburg, MD, USA), Shimadzu Terpene and Terpenoid library (Shimadzu), and Wiley 08 (Wiley, Hoboken, NJ, USA) mass spectral library, as well as the previous data of standards in our lab. Volatile compounds were confirmed to be over 90% with the mass spectral libraries to ensure the searching reliability. Qualification of volatile compounds was also compared with the Kovats retention index of components and previously published data. The Kovats index was calculated based on the following Van den Dool's equation:

$$I = 100z + 100\frac{T(i) - T(z)}{T(z+1) - T(z)}$$

where $T_{(Z)} < T_{(i)} < T_{(Z+1)}$; Z is the number of carbon atoms; $T_{(i)}$ is the retention time of the sample *i*; and $T_{(Z)}$ and $T_{(Z+1)}$ are the retention times of the *n*-alkanes eluted before and after the sample *i*. Quantification of volatiles was conducted and based on the internal standard (6-methyl-5-hepten-2-ol). The standard curve was established in five different concentrations (i.e., 5, 10, 15, 20, and 30 ppm) that were prepared using a purified DCM solvent. Then, 1 μL of the extract was injected into the GC and conducted in triplicate.

Statistical analysis All the samples, i.e., the extracts of samples from stages I, II, III, were conducted in triplicate. PCA and hierarchical clustering were performed using OmicsOffice built in TIBCO Spotfire. PCA was run on log2-transformed area of each chemical compound detected by GC-MS with auto scaling. Hierarchical clustering was performed using complete linkage method with correlation.

Results and Discussion

Identification of VOCs in three different ripening stages In the three ripening stages (i.e., stages I, II, and III shown in Fig. 1) of the Cowart muscadine grapes, 28 VOCs were chromatographically separated and identified by HS-SPME and GC-MS. The qualitative and semi-quantitative data are shown in Table 1. The VOCs are classified into different chemical groups and listed according to their semi-quantitative concentrations (Table 1). During the period of grape maturity, 17 VOCs were tentatively identified in stage I, 12 in stage II, and 25 in stage III. The identified VOCs were categorized into different chemical groups, including alcohols, esters, carbonyl compounds, terpenoids, and norisoprenoids. In stage I, the 17 VOCs included nine terpenoids, four carbonyl compounds, one norisoprenoid, and three alcohols. The 12 VOCs in stage II included five terpenoids, two carbonyl compounds, one norisoprenoid, and four alcohols. The 25 aromas in stage III included eight esters, seven terpenoids, four carbonyl compounds, one norisoprenoid, and five alcohols. The observed chromatographic profiles of muscadine grape flavors in the three different ripening stages seemed to be similar or had differences only in their concentrations. However, in a more detailed analysis, fewer VOCs were detected in stage II. Among these chemical groups, the predominant VOC group in the three ripening stages was the terpenoids, which accounted for 86.82, 75.25, and 40.40% of the extracted volatiles in stages I, II, and III, respectively. This result was similar to a previous report that the terpenoids was the major volatile group of the grape aromas during ripening (16).

Based on our results, esters were only detected in stage III. Esters are a major chemical group found in the fully ripened grapes, and majority of the detected esters were in their acetate forms, which usually result in the fruity, floral, and pleasant aromas of fruits. These aromas seemed to be accumulated during the ripening stages, and were particularly synthesized during the final maturity stage (16,17). The VOCs in the ester group included ethyl acetate, propyl acetate, butyl acetate, ethyl hexanoate, hexyl acetate, ethyl-trans-2-butenoate, butyl-2-butenoate, and hexyl-2-butenoate. Of these, ethyl acetate was the most abundant ester component, accounting for 24.66% of the VOCs present in stage III.

The volatile carbonyl compounds were detected in all three stages

Table 1. Volatile compounds in muscadine grapes were extracted by solid-phase microextraction (SPME), and their concentrations were determined by GC-MS

No	Compounds	Kovats index		Semi-quantitative concentration (ppm)[1)]		
		KI[2)]	KI (Lit)[3)]	STAGE 1	STAGE 2	STAGE 3
	Esters					
1	Ethyl acetate	695	628			61.61±2.41
2	Propyl acetate	737	705			0.37±0.08
3	Butyl acetate	813	816			4.56±0.31
4	Ethyl hexanoate	999	998			0.54±0.13
5	Hexyl acetate	1012	1014			0.32±0.07
6	Ethyl trans-2-butenoate	842	880			2.94±0.11
7	Butyl-2-butenoate	1043				0.58±0.32
8	Hexyl-2-butenoate	1244				0.12±0.04
	Terpenoids					
9	l-limonene	1029	1029	0.76±0.21	0±0.00	0±0.00
10	β-ocimene	1046	1050	0.75±0.21	0±0.00	0±0.00
11	α-terpinolene	1100	1089	61.03±18.14	31.88±5.10	15.28±1.99
12	myrcenol	1122	1123	1.01±0.34	0±0.00	0±0.00
13	ocimenol	1166	1165	4.68±2.65	2.94±0.35	1.15±0.77
14	α-terpineol	1197	1189	33.48±4.86	11.84±0.98	3.82±1.61
15	nerol	1225	1230	1.12±0.39	0.93±0.18	0.49±0.20
16	β-citronellol	1227	1159	1.62±0.66	0±0.00	1.07±0.19
17	trans-geraniol	1253	1253	21.05±5.35	37.41±2.64	78.77±4.30
18	β-citral	1269	1277	0±0.00	0±0.00	0.35±0.16
	Carbonyl compounds					
19	Hexanal	800	801	4.67±1.28	7.17±1.47	11.95±1.52
20	2-hexenal	852	854	9.76±4.56	11.44±2.58	25.23±3.49
21	Nonanal	1105	1101	0.31±0.09	0±0.00	0.15±0.00
22	Decanal	1207	1209	0.1±0.09	0±0.00	0.08±0.05
	Norisoprenoids					
23	β-Damascenone	1386	1384	0.92±0.77	1.57±0.20	5.38±2.21
	Alcohols					
24	*(E)*-2-hexen-1-ol	863	862	0.34±0.10	1.89±0.59	2.21±0.24
25	1-hexanol	867	871	1.14±0.16	4.55±0.90	9.68±0.43
26	1-octanol	1070	1068	0±0.00	0±0.00	3.92±0.45
27	*(Z)*-4-decen-1-ol	1259	1259	0±0.00	0.57±0.07	18.02±0.73
28	1-dodecanol	1479	1474	1.83±0.43	0.81±0.33	1.23±0.47

Values are represented by the meanstandard deviation of three replicate.

[1)]Semi-quantitative concentration calculated from peak area/internal standard peak area internal standard concentration; mean of three replicates± standard deviation.

[2)]The KI index was calculated based on the DB-5MS capillary column and identified using mas spectra database (90% matching similarity).

[3)]Kovat index values that were previously published in literatures.

of berry development. Four compounds, hexanal, 2-hexenal, nonanal, and decanal, were herein categorized into the carbonyl compound group. Carbonyl compounds are major volatile group in fruits and wines (18,19). In particular, hexanal and 2-hexenal were two of the most abundant volatile carbonyl compounds in grapes, and all the carbonyl compounds contribute to the "green or grassy" aromas in grape juices (20). All four volatile carbonyl compounds were detected in the three stages and their odors were easily recognized and differentiated from other odors because their olfactory threshold values are low. Nonanal and decanal were also detected in the Cowart muscadine grapes at stages I and II in very low concentrations. Similar to the volatile esters in grapes, the carbonyl compounds were accumulated to their highest levels in stage III, the fully ripened fruits. 2-Hexenal and hexanal had similar patterns among the different stages. These two C6 compounds have been suggested to form from the lipoxygenase pathway (21). The concentrations of carbonyl compounds have been reported to significantly decrease after stage II (19); however, in this study, concentrations of the carbonyl compounds were found to significantly increase until stage III.

As shown in Table 1, volatile alcohols were also detected, including *(E)*-2-hexen-1-ol, 1-hexanol, 1-octanol, *(Z)*-4-decen-1-ol, and 1-dodecanol. All these chemicals, except 1-octanol and *(Z)*-4-decen-1-ol, were detected in all the three different ripening stages. 1-Octanol was only detected in stage III, while *(Z)*-4-decen-1-ol was detected in both stages II and III. The alcohol concentrations, except that of 1-

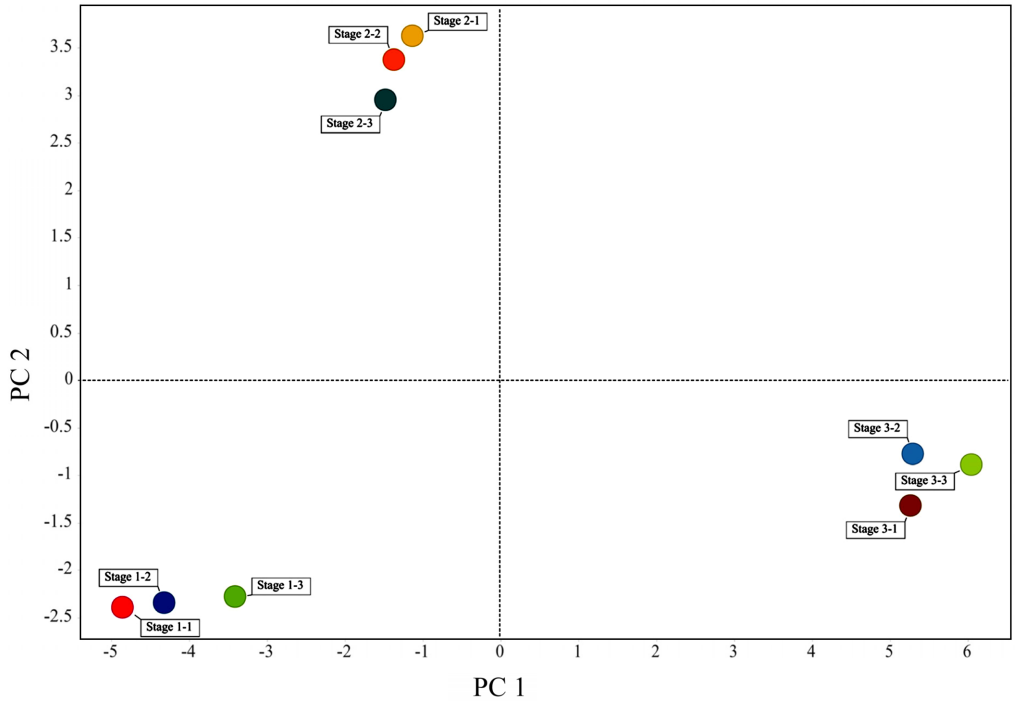

Fig. 2. PCA scatter plots of the volatile compounds detected in three developmental stages of Muscadine grapes (*Vitis rotundifolia*)

dodecnanol that had the highest concentration in stage I, gradually increased until stage III. Owing to the fermentation process that occurs during the grape ripening stages, the alcohol concentrations increased until the harvest time (22). In addition, the 1-hexanol was the second most abundant chemical in the three ripening samples. A previous study reported that 2-phenylethanol, which gives a "rose" aroma, was abundant in muscadine grapes (23), but it was not detected in our study. Such kind of discrepancy was rather difficult to be compared because of many factors, such as different extraction methods (i.e., liquid–liquid extraction vs. SPME), for the determination of the volatile compounds.

Terpenoid VOCs have been intensively studied because they are important for the sensorial differentiation of wines based on grape variety (16). Ten terpenoids, l-limonene, β-ocimene, α-terpinolene, myrcenol, ocimenol, α-terpineol, nerol, β-citronellol, trans-geraniol, and β-citral, were found in the three different ripening stages of muscadine grapes. These VOCs have very pleasant aromas with low olfactory thresholds. Muscadine grapes in stage I had a high concentration of terpenoids. Among them, α-terpinoelene was predominant in this stage and continuously decreased until stage III. On the contrary, trans-geraniol continuously increased during the ripening stages, and finally became the main volatile compound in the last stage.

During the ripening stage, only one C13 norisoprenoid, β-damascenone, was detected in all three ripening stages and its concentration gradually increased through the ripening stages. The norisoprenoids are often found in low concentrations and exist as glycosidic aromas in fruit. β-Damascenone was found by SBSE-GC-MS throughout the screening of three different natural grapes (*Vitis vinifera* L.) of Nebbiolo, Dolcetto, and Barbara. These grapes that contained the lowest amount of β-damascenone (48.2±26.1 to 265.2±83.0 μg/kg) also had a lower level of norisoprenoids than Cowart observed in this study (24). Ristic *et al*. (25) who investigated the C13 norisoprenoid by a solid-phase microextraction GC-MS found that *Vitis vinifera* L. cv. *Shiraz* contained a significantly low concentration of β-damascenone, of which the values ranged from 38.3–71.2 μg/kg. Nevertheless, β-damascenone detected in fruits might be ascribed to the following reason. During the sample preparation steps, the fruits that were crushed in a blender and followed by a moderate thermal heating might induce a pre-fermentative hydrolysis that resulted in the release of free forms of C13 norisoprenoids and facilitate the extraction of VOCs by HS-SPME (26).

Discrimination of VOCs during the ripening stages To examine the similarities of volatile profiles and establish a relationship between the VOCs and three ripening stages of the muscadine grapes, PCA and hierarchical clustering were performed based on the peak areas of VOCs detected by the GC-MS, which are listed in Table 1. Figure 2 shows the principal component (PC) values, which were based on the log2-transformed data of the peak area values of 28 VOCs, wherein 0 was assigned to the chemicals that were not detected by the GC-MS. According to the PCA analysis, 67.75 and 23.53% of the variance were explained by the first principal component (PC1) and second principal component (PC2), respectively. As over 90% of the PCA variance was covered by the first two principal components (PC1

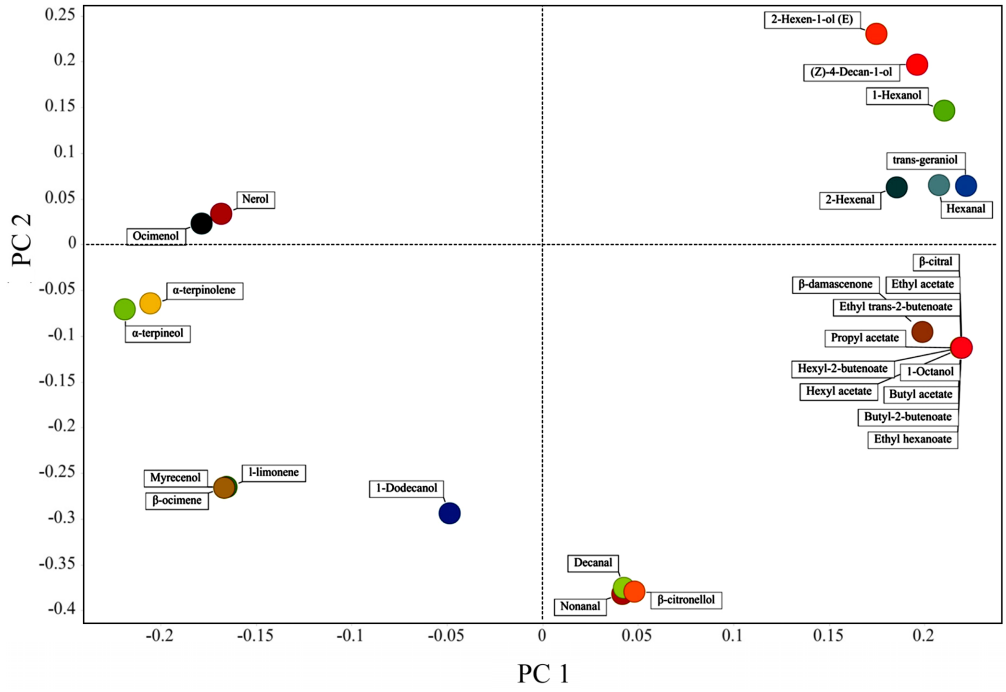

Fig. 3. PCA scatter plots of the individual volatile compounds

and PC2), the aroma profiles of the samples under the three maturity stages were considered to be represented by the two components. The differences among the aromatic profiles of the samples were compared, as shown in Fig. 2. The analyses were performed in triplicate. Figure 2 shows that the samples picked in the same ripen stage were closely clustered together, while the samples of different ripen stages were located in obviously different regions. The average PC1 values of the stage III and I samples had the highest (5.53) and lowest (4.20) values, respectively. The stage II samples had an intermediate average value of PC1 of (1.33). However, the stage II samples had the highest PC2 values of (3.32), while the samples in stage I had the lowest PC2 mean value (2.33) among the three samples. The mean value of PC2 of the stage III samples (1.00) was closer to that of stage I than stage II.

Moreover, the relationship between the individual VOCs within the three maturity stages is profiled in Fig. 3. Based on the aforementioned PCA of the three aroma profiles (shown in Fig. 2), stage III is represented by a positive high PC1 value (5.53) and a negative PC2 value (1.00), which is reflected by and linked to a group of chemical compounds, such as butyl-2-butenoate, hexyl acetate, propyl acetate, ethyl trans-2-butenoate, hexyl-2-butenoate, ethyl acetate, butyl acetate, 1-octanol, ethyl hexanoate, and β-citral, which exhibited highly similar loadings of positive PC1 (0.22) and negative PC2 (0.11) values. This indicated that the aroma pattern (or distribution) of the presence and the amounts of these VOCs was highly correlated. They were correlated with the grape maturity in stage III, which was reflected by their concentration levels. This conclusion is also demonstrated and profiled in Fig. 4, which is a visual representation of the quantification of each volatile compound during the ripening stages. In addition, Figure 4 shows the hierarchical clustering of the VOCs at the three different ripening stages and is useful to further distinguish the VOCs in the three stages, as shown in Fig. 3. For instance, Fig. 4 shows that esters, such as ethyl acetate, propyl acetate, butyl acetate, ethyl hexanoate, hexyl acetate, ethyl trans-2-butenoate, butyl-2-butenoate, and hexyl-2-butenoate, are only produced in stage III. Moreover, three special VOCs, i.e., nonanal, decanal, and β-citronellol, were not detected in the stage II samples (Table 1) and possessed the lowest negative PC2 values (0.38) and low positive PC1 values (0.04–0.05) (Fig. 3). These three VOCs can be easily differentiated from other volatiles in the stage II samples that exhibited the highest PC2 values, as well as the low negative PC1 values (Fig. 2). Therefore, these three VOCs are considered the major markers that distinguish the samples in stages I and III from the sample in stage II that lacks these three volatiles. In conclusion, the outcome shown in Table 1 is consistent with and confirmed by the aroma profiles presented in Fig. 2 and Fig. 3.

There are three terpenoids VOCs, i.e., myrcenol, β-ocimene, and l-limonene, that were detected only in the stage I samples (Table 1). These three volatile terpenoids had similar PC loadings, with both low PC1 and PC2 values. As shown in Fig. 2, the stage I samples, compared with those in stages II and III, had lower average PC1 (4.20) and PC2 (2.33) values, which were obviously distinguished from those of other stages. This outcome coincides with the positional loadings of the PC values of the volatile terpenoids in the stage I samples (Fig. 3), Meanwhile, these terpenoids were lacking in the stage II and III samples. These results suggested that the aforementioned three terpenoids may be major contributors that distinguish the stage I samples from the stage II and III samples.

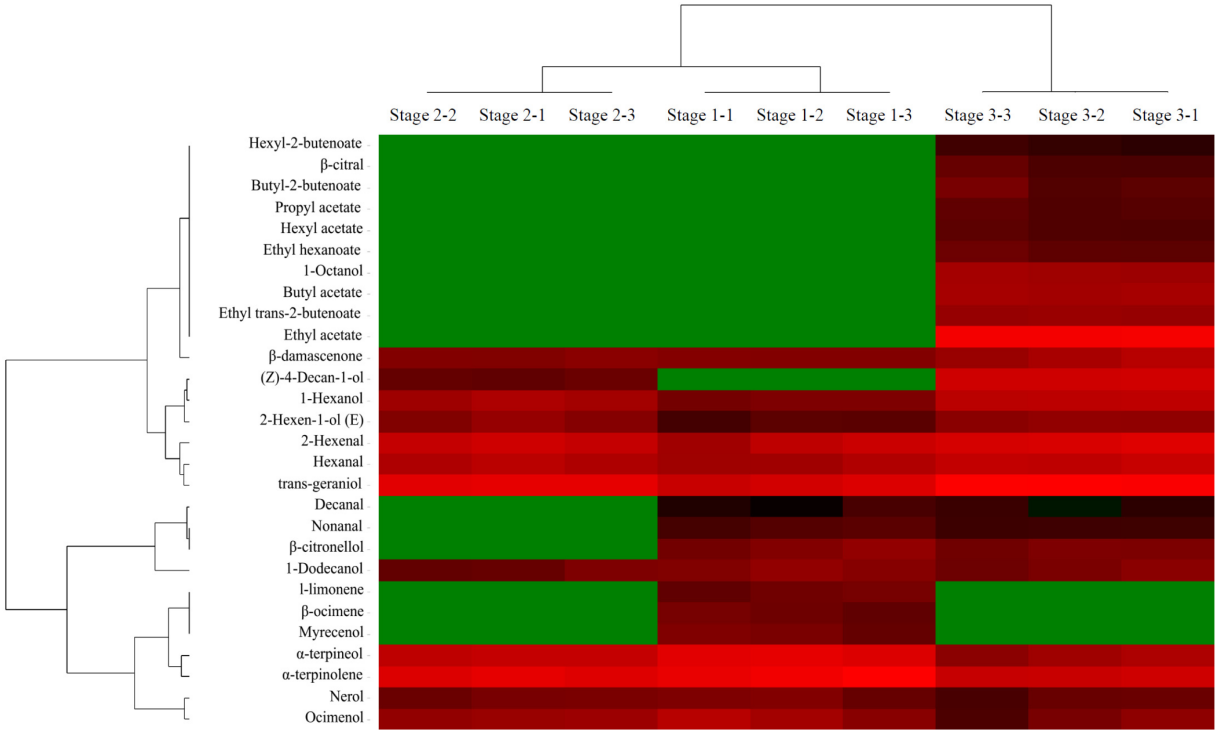

Fig. 4. Hierarchical clustering of volatile compounds at the three different ripening stages (The log2-transformed values of each volatile compound's peak areas, which were detected by GC-MS, are represented by colors. The maximum (24.30), average (12.27), and minimum (0.00) values are represented by red, black, and green, respectively.

It was also observed that three alcohols, including *(E)*-2-hexen-1-ol, *(Z)*-4-decen-1-ol, and 1-hexanol, had both high positive PC1 and PC2 values that were significantly different from those of other chemicals. However, these three volatiles existed in null or relatively low concentrations in stage I, implying that the stage I samples might also be differentiated by these three volatile chemicals, particularly *(Z)*-4-decen-1-ol, which was clearly different from the chemicals shown in Fig. 4. Furthermore, the hierarchical clustering (Fig. 4) was conducted to find the inter-connectivity, as well as the closeness of individual volatiles. It revealed the similarities between the individual volatiles and their contributions to the aroma profiles in the three ripening stages. Each VOC, as a log2-transformed value of the peak area from GC-MS, is represented by a color in the heat map. Maximum (24.30), average (12.27), and minimum (0.00) values are represented by red, black, and green, respectively.

Hierarchical clustering (Fig. 4) shows that butyl-2-butenoate, hexyl acetate, propyl acetate, ethyl trans-2-butenoate, hexyl-2-butenoate, ethyl acetate, butyl acetate, 1-octanol, ethyl hexanoate, and β-citral were clustered very closely in one group. However, myrcenol, β-ocimene, and l-limonene were closely grouped together in the hierarchical clustering. In addition, *(E)*-2-hexen-2-ol, *(Z)*-4-decen-1-ol, and 1-hexanol, which were distinct chemicals in the samples of stage I and had relatively low concentrations compared with in samples from the other stages, were found closely clustered. Nonanal, decanal, and β-citronellol were not abundant in stage II. They were also found to be clustered together. These hierarchical clustering classes, which clustered the chemical compounds based on abundance patterns, were similar to the patterns of the PCA results (Fig. 2 and Fig. 3).

Overall, muscadine grapes collected in three ripening stages were investigated and 28 VOCs were identified. PCA of these VOCs revealed that grapes in the three different ripening stages had significantly different aroma patterns, particularly in terms of concentrations of the VOCs, although they had subtle differences in their chemical compositions. Butyl-2-butenoate, hexyl acetate, propyl acetate, ethyl trans-2-butenoate, hexyl-2-butenoate, ethyl acetate, butyl acetate, 1-octanol, ethyl hexanoate, and β-citral were distinct VOCs that were only detected in stage III. Nonanal, decanal, and β-citronellol formed another group of distinct chemicals that were absent in the stage II samples; thus, they could be used as chemical markers to differentiate the stage II samples from other samples. Terpenoids, which were accumulated during the ripening stages, are another group of biomarkers that can be used to identify the maturity stage. Although α-terpinolene and trans-geraniol were the predominant VOCs in muscadine grapes, myrcenol, β-ocimene, and l-limonene were detected only in the stage I samples. In contrast with the terpenoids, volatile esters, which are associated with fruity, floral, and pleasant odors were only detected in the fully ripened grapes in stage III. Thus, the grapes in stage III were highly favorable for fresh consumption or made into desirable wines because of their desirable and rich aromas.

Disclosure The authors declare no conflict of interest.

References

1. Barchenger DW, Clark JR, Threlfall RT, Howard LR, Brownmiller CR. Nutraceutical changes in muscadine grape and grape segments during storage. J. Amer. Pomolog. Soc. 69: 66-73 (2015)
2. Kim TJ, Silva JL, Weng WL, Chen WW, Corbitt M, Jung YS, Chen YS. Inactivation of *Enterobacter sakazakii* by water-soluble muscadine seed extracts. Int. J. Food Microbiol. 129: 295-299 (2009)
3. Perestrelo R, Barros AS, Rocha SM, Câmara JS. Establishment of the varietal profile of *Vitis vinifera* L. grape varieties from different geographical regions based on HS-SPME/GC–qMS combined with chemometric tools. Microchem. J. 116: 107-117 (2014)
4. Sánchez-Palomo E, Diaz-Maroto MC, Perez-Coello MS. Rapid determination of volatile compounds in grapes by HS-SPME coupled with GC–MS. Talanta 66: 1152-1157 (2005)
5. López-Tamames E, Carro-Mariño N, Gunata YZ, Sapis C, Baumes R, Bayonove C. Potential aroma in several varieties of Spanish grapes. J. Agr. Food Chem. 45: 1729-1735 (1997)
6. Mamede ME, Pastore GM. Study of methods for the extraction of volatile compounds from fermented grape must. Food Chem. 96: 586-590 (2006)
7. Noguerol-Pato R, González-Álvarez M, González-Barreiro C, Cancho-Grande B. Simal-Gándara J. Evolution of the aromatic profile in *Garnacha tintorera* grapes during raisining and comparison with that of the naturally sweet wine obtained. Food Chem. 139: 1052-1061 (2013)
8. Verzera A, Ziino M, Scacco A, Lanza CM, Mazzaglia A, Romeo V, Condurso C. Volatile compound and sensory analysis for the characterization of an Italian white wine from "Inzolia" grapes. Food Anal. Method. 1: 144-151 (2008)
9. Lee JH, Talcott ST. Fruit maturity and juice extraction influences ellagic acid derivatives and other antioxidant polyphenolics in muscadine grapes. J. Agr. Food Chem. 52: 361-366 (2004)
10. Dokoozlian NK. Grape berry growth and development. Raisin Production Manual. University of California, Agricultures & Nautral Resources Publications, Oakland, CA, USA. pp. 30-37 (2000)
11. Palomo ES, Díaz-Maroto M, Viñas MG, Soriano-Pérez A, Pérez-Coello M. Aroma profile of wines from Albillo and Muscat grape varieties at different stages of ripening. Food Control 18: 398-403 (2007)
12. Vilanova M, Genisheva Z, Bescansa L, Masa A, Oliveira JM. Changes in free and bound fractions of aroma compounds of four *Vitis vinifera* cultivars at the last ripening stages. Phytochemistry 74: 196-205 (2012)
13. Yang C, Wang Y, Wu B, Fang J, Li S. Volatile compounds evolution of three table grapes with different flavor during and after maturation. Food Chem. 128: 823-830 (2011)
14. Fan W, Xu Y, Jiang W, Li J. Identification and quantification of impact aroma compounds in 4 nonfloral *Vitis vinifera* varieties grapes. J. Food Sci. 75: S81-S88 (2010)
15. Wejnerowska G, Gaca J. Application of headspace solid-phase microextraction for determination of chloro-organic compounds in sewage samples. Toxicol. Mech. Method. 18: 543-550 (2008)
16. Salinas M, Zalacain A, Pardo F, Alonso GL. Stir bar sorptive extraction applied to volatile constituents evolution during *Vitis vinifera* ripening. J. Agr. Food Chem. 52: 4821-4827 (2004)
17. Kalua CM, Boss PK. Evolution of volatile compounds during the development of Cabernet Sauvignon grapes (*Vitis vinifera* L.). J. Agr. Food Chem. 57: 3818-3830 (2009)
18. Slegers A, Angers P, Ouellet , Truchon T, Pedneault K. Volatile compounds from grape skin, juice and wine from five interspecific hyb frid grape cultivars grown in Quebec (Canada) for wine production. Molecules 20: 10980-11016 (2015)
19. Garcia E, Chacón J, Martínez J, Izquierdo P. Changes in volatile compounds during ripening in grapes of Airén, Macabeo and Chardonnay white varieties grown in La Mancha region (Spain). Food Sci. Technol. Int. 9: 33-41 (2003)
20. Yang C, Wang Y, Liang Z, Fan P, Wu B, Yang L, Wang Y, Li S. Volatiles of grape berries evaluated at the germplasm level by headspace-SPME with GC–MS. Food Chem. 114: 1106-1114 (2009)
21. El Hadi MAM, Zhang FJ, Wu FF, Zhou CH, Tao J. Advances in fruit aroma volatile research. Molecules 18: 8200-8229 (2013)
22. Jordão AM, Vilela A, Cosme F. From sugar of grape to alcohol of wine: Sensorial impact of alcohol in wine. Beverages 1: 292-310 (2015)
23. Lamikanra O, Grimm CC, Inyang ID. Formation and occurrence of flavor components in Noble muscadine wine. Food Chem. 56: 373-376 (1996)
24. Carlomagno A, Schubert A, Ferrandino A. Screening and evolution of volatile compounds during ripening of 'Nebbiolo', 'Dolcetto', and 'Barbera' (*Vitis vinifera* L.) neutral grapes by SBSE–GC/MS. Eur. Food Res. Technol. 242: 1-13 (2016)
25. Ristic R, Bindon K, Francis L, Herderich M, Iland P. Flavonoids and C13-norisoprenoids in *Vitis vinifera* L. cv. Shiraz: Relationships between grape and wine composition, wine color and wine sensory properties. Aust. J. Grape Wine. R. 16: 369-388 (2010)
26. Coelho E, Rocha SM, Barros AS, Delgadillo I, Coimbra MA. Screening of variety-and pre-fermentation-related volatile compounds during ripening of white grapes to define their evolution profile. Anal. Chim. Acta 597: 257-264 (2007)