

Green Odorants of Grape Cluster Stem and Their Ability To Cause a Wine Stemmy Flavor

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Five aliphatic carbonyl compounds and two methoxy-pyrazines (MPs) were identified as olfactory dominant green odorants in macerated grape cluster stems of *Vitis vinifera* var. Cabernet Sauvignon and Chardonnay by a gas chromatography eluate sniffing and dilution analysis technique. Quantitative analysis of these odorants and related volatiles showed that the MP content of the stems was higher than those of the berry or leaf samples, though the aliphatic carbonyl compound content of the stems was not as high as the other parts. The addition of blended stems to the vinification process increased the MP content of the wine, but the identified aliphatic green odorants of the macerated stems were not detected in the wine.

Keywords: Grape stem odorant; Cabernet Sauvignon; Chardonnay; methoxy-pyrazine; stemmy flavor; *Vitis vinifera*; wine

INTRODUCTION

Although grape cluster stems are usually removed during vinification, if flavor or taste components of stems are favorable for red wine styles, partial or whole stems are retained. Stems are also involved in a vinification process as a result of undesirable incomplete destemmings. The mixed stems provide some woody herbaceous aromas to the wine that are reminiscent of herbaceous, peppery, bitter components and tannin and can also cause a significant color loss, so they are considered to be of little value or a negative factor (Boulton et al., 1995). Some green odorants have been found as off-flavors of wine in the course of extensive studies of volatile compounds in grapes and wines (Webb and Muller, 1972; Rapp, 1988), but the odorants of the stemmy flavor of wine have not been studied.

The aliphatic C₆ compounds, well-known as green odorants of plants, have been reported in grapes and wines by a number of investigators (Webb and Muller, 1972). The C₆ aldehydes are formed very rapidly during grape crushing in the presence of air by the enzymatic oxidation of unsaturated lipids (Cayrel et al., 1983; Roufet et al., 1986). The formed C₆ aldehydes, the most abundant volatiles of macerated grape leaves, are rapidly converted to hexanol, a process that is accelerated in the presence of yeast (Joslin and Ough, 1978). In addition to the C₆ compounds, the presence of other grassy odorants was reported in Chenin blanc grape leaves by Wildenrath et al. (1975). They observed five major grassy aroma areas in the chromatogram of the extract by a gas chromatography–sniffing analysis but did not identify the compounds contributing to the grassy aroma areas.

Recently, we found that the level of three MP compounds in the Cabernet Sauvignon grape cluster stems was higher than in the other parts of the grapes (Hashizume and Umeda, 1996). Isobutyl and isopropylMP, which have an herbaceous or grassy aroma and extremely low sensory detection thresholds, are present in trace quantities in certain variety grape musts and

wines, playing an important role in the aromatic characteristics of the grape wines (Lacey et al., 1991; Allen et al., 1991, 1994; Allen and Lacey, 1993).

The purpose of this study was to identify the olfactory dominant green odorants of macerated grape cluster stems by an aroma extract dilution analysis (AEDA) technique and also to investigate the abilities of the odorants to cause a wine stemmy flavor.

MATERIALS AND METHODS

Materials. Grapes of *Vitis vinifera* var. Cabernet Sauvignon and Chardonnay were obtained from a local vineyard at Kitakoma, Yamanashi prefecture. Chardonnay grapes whose must composition was Brix, 19.9, titratable acidity as tartaric acid, 7.8 g/L, pH 3.44, were harvested September 5, 1995. Cabernet Sauvignon grapes whose must composition was Brix, 18.3, titratable acidity as tartaric acid, 10.4 g/L, pH 3.49, were harvested October 3, 1995. Grape leaves of the two cultivars were obtained September 20, 1995, from our vineyard at Higashihiroshima, Hiroshima prefecture. All samples were frozen rapidly and stored at –85 °C until needed for analysis. (*Z*)-1,5-Octadien-3-one and (*Z*)-3-hexenal were synthesized according to Swoboda and Peers (1977) and Kajiwara et al. (1975). Suppliers of the standard compounds were as follows: 3-hexanone, 4-nonanol, nonanol, (*E*)-2-nonenal, (*E*)-2-nonenol, (*E,Z*)-2,6-nonadienol, and dodecanal, Tokyo Kasei Kogyo Co. (Tokyo, Japan); hexanal, (*E*)-2-hexenal, hexanol, (*Z*)-3-hexenol, (*E*)-2-hexenol, and nonanal, Wako Pure Chemical Industries (Osaka, Japan); (*E*)-3-hexenol, (*E,Z*)-2,6-nonadienal, and pyrazine compounds, Aldrich Chemical Co.

Isolation of Volatiles for AEDA. Frozen grape cluster stems were ground with a mortar and pestle with liquid nitrogen. The resultant powder (20 g) was mixed with 20 mL of warm water. The suspension was incubated at 25 °C for 15 min to promote the formation of green odorants, and then mixed with 40 mL of an aqueous saturated CaCl₂ solution, which was used by Buttery et al. (1988) in a study on the aroma compounds of tomatoes, to inhibit further enzymatic reactions. After blending, the suspension was centrifuged for 30 min at 25000g and 0 °C. The supernatant was filtered with a filter paper that had previously been washed with dichloromethane. The filtrate was blended with 40 mL of water to decrease the specific gravity and then extracted twice with 20 mL of dichloromethane. The combined dichloromethane was dried over sodium sulfate and concentrated to 100 μL under nitrogen gas.

Gas Chromatography AEDA. The volatiles were separated by capillary gas chromatography on a 0.25 μm DB-WAX

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(J&W Scientific) column (30 m × 0.32 mm) and Shimadzu GC-17A gas chromatograph. The volatile sample (5.0 μ L) was injected using the splitless and programmed temperature vaporizer mode. The injection vaporizer temperature was increased from 50 to 250 °C at a rate of 250 °C/min and then held at that temperature for 10 min. The oven temperature was held at 50 °C for 3 min and then increased at a rate of 6 °C/min to 240 °C. Helium was used as a carrier gas with a flow rate of 2.3 mL/min. The odors were perceived at the end of the column which was held at 200 °C. The aromagrams of the green odorants were evaluated by three judges and plotted according to Ullrich and Grosch (1987, 1988).

Identification Experiments. Mass spectrometry was performed with a Hewlett Packard 6890 gas chromatograph coupled to a Hewlett Packard 6890 MSD. Extract samples for mass spectrometry were prepared from the same procedure for quantification analyses. The following capillary columns were used: (i) 0.25 μ m CPB20 which is equivalent to Carbowax 20M (Shimadzu, Kyoto, Japan), 50 m × 0.2 mm; (ii) 0.33 μ m Ultra-1 (Hewlett Packard), 50 m × 0.2 mm. The injector temperature was 150 °C, and the splitless mode was used. Injection volumes ranged from 0.5 to 2.0 μ L, depending on sample. The oven was programmed at 4 °C/min from 40 to 240 °C and then held for 10 min. The transfer line temperature was held at 260 °C. The flow rate of the carrier gas (helium) was 1.0 mL/min. The mass spectrometer was operated with an ionization voltage of 70 eV and was scanned from m/z 40 to 300 at 0.57 s/scan. The mass spectra were verified by comparison with authentic compounds on the same instrument. The odorants olfactory detected on a DB-WAX column by AEDA were also confirmed using the retention index (RI) and odor characteristics on the Ultra-1 column. The agreement of the RI data with authentic compounds were confirmed by coelution analyses.

Isolation of Volatiles for Quantification. C₆ compounds and dodecanal were isolated from 40 g of berries or 20 g of stems or leaves, with the exception of 1 g of leaves for (*Z*)-3-hexenal determination, by the isolation method used for the AEDA. After the addition of 40 mL of a saturated CaCl₂ solution, 3-hexanone (100 μ g) and 4-nonanol (20 μ g) in an ethanol solution were added as internal standards (IS) to the suspension. Wine (80 mL) was mixed with the IS solution and then analyzed in the same manner as the filtrates of the grape samples. The dichloromethane extracts were concentrated to 1 mL under nitrogen gas. (*Z*)-1,5-Octadien-3-one and C₉ compounds were isolated from 120 g of berries or 60 g of stems or leaves. The frozen samples were cracked with liquid nitrogen and then mixed with 2 (berries) or 3 (stems or leaves) volumes of water. The suspension was homogenized for 5 min and then incubated at 25 °C for 10 min, except for leaf sample which was incubated for 55 min to decrease (*Z*)-3-hexenal because a large amount of the formed (*Z*)-3-hexenal interfered with the quantification of the other volatile compounds. After the addition of an equal volume of a saturated CaCl₂ solution, the suspension was mixed with 4-nonanol (20 μ g) as an IS and then centrifuged to obtain a supernatant. The filtrated supernatant and wine (240 mL) that was mixed with an IS solution were placed on a column of 2 g of Mega Bond Elute C18 (Varian) which was conditioned by methanol (20 mL) and then water (20 mL) successively. After the column was washed by water (20 mL), the trapped volatiles were eluted with 7 mL of dichloromethane. The dichloromethane solution was washed with 1 M Na₂CO₃ solution, dried over sodium sulfate, and then concentrated to about 20 μ L under nitrogen gas. Samples from vine tissues were prepared in duplicate.

Quantification. Gas chromatography mass spectrometry was performed under the same conditions as the identification procedure, except the injection temperature was 200 °C. Target compounds were identified by a scan mode analysis, and then re-analyzed with a selected ion monitoring mode by using the characteristic mass fragments which had relatively high m/z values and less interference by the other compounds. The fragments (m/z) used to quantification were as follows: hexenal, 56; (*Z*)-3-hexenal, 69; (*E*)-2-hexenal, 98; hexanol, 56; (*E*)-3-hexenol, 55; (*Z*)-3-hexenol, 67; (*E*)-2-hexenol, 57; 1,5-octadiene-3-one, 55, 95, or 109; nonanal, 95, 96, or 98; (*E,Z*)-

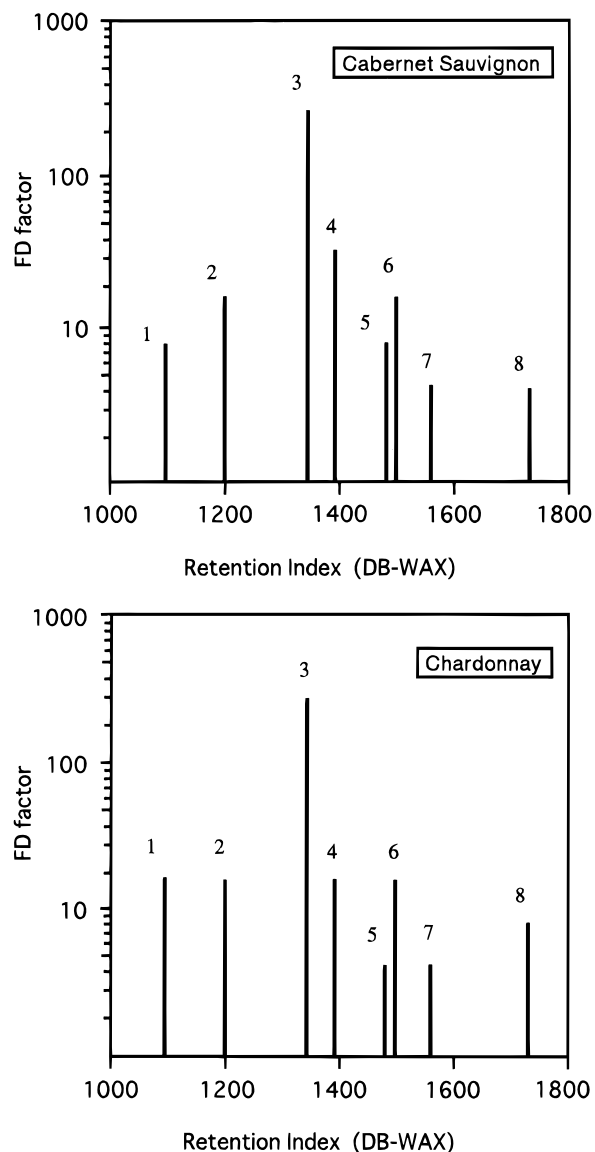


Figure 1. Aromagram of the green odorants isolated from macerated grape cluster stems. Numbering of the odorants as Table 1. "FD factor" is the flavor dilution factor.

2,6-nonadienal, 70 or 94; (*E*)-2-nonenal, 70 or 83; (*E,Z*)-2,6-nonadienol, 93 or 109; (*E*)-2-nonenol, 82, 95, or 96; nonanol, 97 or 98; and dodecanal, 96, 109, or 140. Samples were quantified using calibration curves, which were prepared from the standard solutions at four concentration levels: C₆ compounds, ranging from 0.25 to 10 mg/L; dodecanal, from 25 to 1000 μ g/L; (*Z*)-1,5-octadien-3-one and C₉ compounds, from 8 to 400 μ g/L of a 0.5% (w/v) tartaric acid solution with pH adjusted to 3.5. The solutions were analyzed in the same way as that applied to the incubated homogenate solutions of plant tissues. The ratio between the peak areas of the selected m/z of the measured compound and that of the IS (3-hexanone, m/z 100; 4-nonanol, m/z 101) was used. The calibration curves were linear [e.g., (*E*)-2-hexenal, $r^2 = 0.9991$, weight ratio from 0.1 to 4.0]; (*E,Z*)-2,6-nonadienal, $r^2 = 0.9987$, weight ratio from 0.048 to 2.4], and the estimated detection limits for C₆ compounds and dodecanal were less than 10 μ g/L of the homogenate solutions and less than 5 μ g/L of wine and less than 1 and 0.5 μ g/L, respectively, for (*Z*)-1,5-octadien-3-one and C₉ compounds. MP compounds were analyzed by a previously reported method (Hashizume and Umeda, 1996), whose estimated detection limit for isobutylMP was less than 0.5 ng/L of wine and less than 1 ng/L for isopropylMP and *sec*-butylMP.

Fermentation. The thawed berries (1.5 kg) were crushed by hand and then put into a glass vessel. After the addition

Table 1. Green Odorants^a Detected in the Grape Cluster Stems by AEDA and Results of Identification^b

aromagram no.	compound	retention index (RI)		odor description
		DB-WAX	Ultra-1	
1	hexanal	1099	800	green
2	(<i>E</i>)-2-hexenal	1200	844	green
3	(<i>Z</i>)-1,5-octadien-3-one	1346	963	geranium-like, metallic green
4	2-methoxy-3-isopropylpyrazine	1394	1092	grassy, earthy
5	unknown	1484	—	cucumber-like
6	2-methoxy-3-isobutylpyrazine	1500	1211	herbaceous, earthy
7	(<i>E,Z</i>)-2,6-nonadienal	1561	1150	cucumber-like
8	dodecanal	1737	1402	citrus skin-like

^a Green, vegetative, or herbaceous odorants showing flavor dilution factors ≥ 4 were analyzed. ^b Identified by MS/EI, RI, and odor quality.

Table 2. Aliphatic Green Volatile Compounds^a in the Macerated Grape Samples

compound	aromagram no.	Cabernet Sauvignon			Chardonnay		
		stem	berry	leaf	stem	berry	leaf
hexanal	1	520	2.4×10^3	5.3×10^3	1.9×10^3	1.9×10^3	9.6×10^3
(<i>Z</i>)-3-hexenal			910	2.2×10^5		220	2.1×10^5
(<i>E</i>)-2-hexenal	2	5.7×10^3	8.0×10^3	1.7×10^4	9.5×10^3	4.8×10^3	1.2×10^4
hexanol		230	300	160	740	350	43
(<i>E</i>)-3-hexenol		39		15	42	13	7
(<i>Z</i>)-3-hexenol		160	34	330	180	28	520
(<i>E</i>)-2-hexenol		840	670	500	1.2×10^3	610	310
(<i>Z</i>)-1,5-octadien-3-one	3	2		12	2		12
nonanal		480	99	260	470	190	370
(<i>E,Z</i>)-2,6-nonadienal	7	5	1		3	3	
(<i>E</i>)-2-nonenal		20	5		19	9	
(<i>E,Z</i>)-2,6-nonadienol							
(<i>E</i>)-2-nonenol							
nonanol		100	28		120	100	
dodecanal	8	240	34	420	190	13	360

^a Nanograms per gram of samples. Average of two determinations.

of potassium bisulfite up to 200 ppm, an activated wine yeast V-1116 (Lallemand Inc.) was inoculated to the must. The stems, obtained from 1.5 kg of the grape clusters, were blended slightly using a Waring Blender to simulate wine production conditions, and then added to the experimental section must. Fermentation was conducted with skin at 25 °C for 7 days in duplicate. The fermented must was pressed at the seventh day. The wine was centrifuged at 17400g, and the supernatant was used for analysis.

RESULTS AND DISCUSSION

AEDA and Green Odorant Identification from Stems. The incubation time of the volatile isolation procedure was determined as a result of time-course experiments about the generation and fate of C₆ aldehydes in the macerated stems and berries, from which the preservation of the enzymatic activities of the frozen samples and the inhibitory action of CaCl₂ to the enzymatic reaction were also confirmed. The amount of (*E*)-2-hexenal, which was the most prevalent C₆ aldehyde in the macerated stems, became maximal from 10 to 20 min after incubation started.

The aromagram of each cultivar (Figure 1) showed eight dominant green odorants with FD factors of 4 and higher, respectively. The eight odorants of each cultivar were identified as the same compounds. In addition to these green odorants, a cooked vegetable-like odorant that was suggestive of a sulfur-containing compound (RI on the DB-WAX column was 1318), a burnt bamboo-like odorant (1605), and a sweaty unpleasant odorant (1651) were detected in both cultivars, and a floral aroma (1959) was detected only in the volatiles of Cabernet Sauvignon, but these odorants were not analyzed further because the stemmy flavor belongs to a fresh vegetative aroma group (Noble et al., 1987). The results of the identification experiments are sum-

marized in Table 1. Compounds 1 and 2 were C₆ aldehydes which have been reported in grape berries and leaves (Drawert et al., 1965; Stevens et al., 1966; Hardy, 1970). Compound 3, having an intense geranium-like or metallic-green odor, showed the highest FD factor. It was identified as (*Z*)-1,5-octadien-3-one, a compound that was reported first as an autoxidation product of butterfat (Swoboda and Peers, 1977) but has never been reported in grape samples. Schieberle et al. (1990) found that the odorant was a aroma constituent of the volatiles from homogenized cucumber and muskmelon. The odorant was quite different from 2-ethoxyhexa-3,5-diene reported as a source of geranium-like off-odor of wine (Crowell and Guymon, 1975). Aroma-active peaks 4 and 6, identified as isopropylMP and isobutylMP, showed about the same FD factors. We could not identify compound no. 5, but it was believed to be a C₉ aldehyde because of its RI and odor quality. In this AEDA, we could not detect terpenes or their related compounds which might contribute to the green notes of grape stems, though α -terpineol and geraniol were identified in the isolated volatiles.

Quantitative Results of Green Odorants in Vine Plants. The amounts of the aliphatic green odorants identified in the AEDA and their related volatiles in the macerated stem, berry, and leaf samples are shown in Table 2. In the crushed plant tissues, the formation of C₆ aldehydes was affected by the enzymatic activity which varied significantly with reaction conditions (Joslin and Ough, 1978) and by the physiological or environmental conditions of the plant (Sekiya et al., 1984), therefore, the results showed only a part of their profiles, but we thought the relative comparisons of the compounds to be significant for this investigation. The remarkable difference in the amounts of the C₆ alde-

Table 3. Methoxypyrazine Compounds^a in the Grape Samples

compound	aromagram no.	Cabernet Sauvignon				Chardonnay			
		stem	juice	skin + seed	leaf	stem	juice	skin + seed	leaf
2-methoxy-3-isopropylpyrazine	4	43.0		1.4		69.3		2.5	
2-methoxy-3-sec-butylpyrazine		64.0		4.8		100.4		5.4	
2-methoxy-3-isobutylpyrazine	5	205.0	17.5	130.1	21.8	275.8	5.3	83.7	47.3

^a Nanograms per kg of samples. Average of two determinations.

Table 4. Green Volatile Compounds in the Wines Fermented with Skins

compound	aromagram no.	Cabernet Sauvignon		Chardonnay	
		- stem	+ stem	- stem	+ stem
aliphatic compounds ^a					
hexanal	1				
(Z)-3-hexenal					
(E)-2-hexenal	2				
hexanol		2.4×10^3	2.7×10^3	2.7×10^3	2.8×10^3
(E)-3-hexenol		190	230	170	170
(Z)-3-hexenol		72	100	200	200
(E)-2-hexenol		4	8	9	22
(Z)-1,5-octadien-3-one	3				
nonanal					
(E,Z)-2,6-nonadienal	7				
(E)-2-nonenal					
(E,Z)-2,6-nonadienol					
(E)-2-nonenol					
nonanol		12	6	21	19
dodecanal	8				
methoxypyrazine compounds ^b					
2-methoxy-3-isopropylpyrazine	4		2.7 ± 0.29		2.5 ± 0.35
2-methoxy-3-sec-butylpyrazine			2.8 ± 0.24		2.0 ± 0.44
2-methoxy-3-isobutylpyrazine	5	25.3 ± 2.10	33.8 ± 1.62	11.6 ± 0.59	18.0 ± 0.73

^a Nanograms per mL of wine. Average of two determinations. ^b Nanograms per liter of wine. Mean and standard deviation of four determinations.

hydes among the sample parts might be due to the distribution of enzymatic activities for C₆ aldehyde formation (Sekiya et al., 1983). (Z)-1,5-Octadien-3-one, showing the highest FD factor in the AEDA, was detected in both the stem and the leaf parts but not in the berries. The three MP measured in both grape stems were higher than in the other parts of the plant (Table 3), therefore these MP compounds seemed to be the characteristic aroma constituents of the two cultivar's stem parts. IsobutylMP contents were five times higher than isopropylMP contents in the stems of both cultivars, though the DF values of the two MPs were about the same level. This result might be due to a difference of odor threshold values of these two MP compounds assessed in the AEDA system as reported by Chisholm et al. (1995).

Green Odorants in Wines. The concentrations of the volatile compounds in the wines fermented with skins are shown in Table 4. We could not detect in the wines any aliphatic carbonyl compounds which were detected in the macerated grapes, though some C₆ aldehydes have previously been detected in the wines (Webb and Muller, 1972; Chisholm et al., 1994, 1995). Hexanol in the wines was quite higher than the used grapes, and it was suggested that some of the C₆ aldehydes and unsaturated alcohols were converted to hexanol as reported by Herraiz et al. (1990). The addition of the blended stems to the vinification processes did not affect the aliphatic compound contents in the wines, except for slight increases of hexanol and (E)-2-hexenol. Changes of the spiked (Z)-1,5-octadien-3-one and C₉ aldehydes in the vinification processes were investigated to clarify the behaviors of the compounds (Figure 2) that have very low thresholds and exist at trace levels in the grapes. The compound amounts were decreased immediately after the addition of SO₂ and

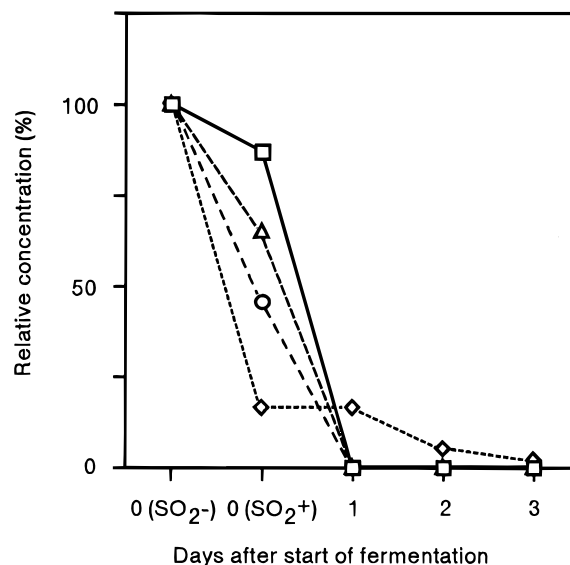


Figure 2. Changes in relative concentrations of the spiked (Z)-1,5-octadien-3-one (\square), nonanal (\diamond), (E,Z)-2,6-nonadienal (\circ), and (E)-2-nonenal (\triangle) during early vinification process. Amounts of each spiked compound at the starting point were (Z)-1,5-octadien-3-one, 101 μg ; nonanal, 1352 μg ; (E,Z)-2,6-nonadienal, 648 μg ; and (E)-2-nonenal, 722 μg .

disappeared in an early period of the fermentation. It is supposed that some of these carbonyl compounds reacted with SO₂ rapidly, and some of the rest compounds might be reduced to alcohols as like as C₆ aldehydes, but the low level of nonanol in the wines indicated further conversions during fermentation. On the other hand, the blended stems distinctly increased the MP contents of the wines. IsopropylMP was detected only in the treated wines, and the isopropylMP levels were above the sensory detection threshold in a

water solution (Seifert et al., 1970). Recently, Allen et al. (1995) reported on the isopropylMP contents of Bordeaux red wines and discussed the reason for the extraordinarily high isopropylMP content (about 10 ng/L) in the red wine. Although we did not find such a large amount of isopropylMP in the stem-blended wines, the stems, if they were vinified with grape berries, might be able to supply a certain level of isopropylMP to the wine.

Among the analyzed green odorants of macerated stems of the two cultivars, MP compounds were abundant in stem parts and also remained in the wines after fermentation, and the increased amounts of MP compounds by the addition of stem seemed to provide a sensory effect to the wines, therefore it seems likely that the MP compounds in the grape stems are capable of causing a stemmy flavor of wine.

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