Dynamic Changes in Volatile Compounds during Fermentation of Cabernet Sauvignon Grapes with and without Skins

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Abstract: Volatile compounds in wines arise from many sources: they may be extracted from the grapes during fermentation, formed by yeast during fermentation, and/or arise during postfermentation storage and processing. Factors influencing extraction of volatiles from grapes during fermentation have not been widely studied; an improved understanding of the processes and mechanisms involved in the formation or release of wine aroma compounds from grapes during fermentation could help winemakers to optimize or control wine composition and aroma. In this work, we studied the effects of different skin contact times on changes in concentrations of volatile aroma compounds during fermentation of Cabernet Sauvignon grapes. Headspace solid-phase microextraction coupled to gas chromatography-mass spectrometry was used to monitor the volatile compounds produced during fermentation. Duration and timing of the skin contact during fermentation showed a measurable effect on volatile composition. Some compounds reached higher concentrations in fermentations performed with skins, while, in other cases, skins acted to "bind" or delay release of volatiles, particularly β-damascenone.

Key words: wine, Cabernet Sauvignon, HS-SPME GC-MS, β-damascenone, C_{13}-norisoprenoids, acetate esters, fatty acid ethyl esters, C_6-aldehydes, C_6-alcohols

Aroma is one of the most important indicators of wine quality, leading winemakers to choose grapes with optimal flavor and aroma and to optimize fermentation and processing conditions to increase the aroma quality of the final wines (Rapp 1988). In general, compounds contributing to wine aroma have three different origins: compounds which derive from the grape, compounds formed during fermentation (produced by yeast or by non-*Saccharomyces* microbial growth), and compounds extracted from oak barrels or formed during the aging process (Polaskova et al. 2008). Volatile compounds derived from grapes include alcohols, esters, acids, terpenes, norisoprenoids, polyfunctional thiols, and carbonyl compounds (Polaskova et al. 2008). More than half of the total volatile aroma compounds present in grape berries are localized in the exocarp (skin) tissue and many are typically stored as sugar or amino acid conjugates in the vacuoles of the exocarp cells (Seymour et al. 1993, Gomez et al. 1994, Fernández et al. 2000). Therefore, skin contact time during fermentation is one variable that can be modified during the winemaking process to influence final wine composition. Longer duration of skin contact during fermentation and postfermentation extended maceration of red wines significantly increases levels of phenolic compounds in the final wines (Zimman et al. 2002, Sacchi et al. 2005). Effects on volatile composition have not been as well studied.

Volatile aroma compounds in grapes are released into the finished wine by several chemical and physical mechanisms. Some of the aroma compounds that exist in their "free" volatile state in grape juice are extracted into the wine following crushing/maceration of the skins and do not undergo further modifications during fermentation (e.g., 3-isobutyl-2-methoxypyrazine) (Kotseridis and Baumes 2000). Nonvolatile precursors, including glycosides and cysteinylated/glutathionylated compounds, are extracted from the grapes and hydrolyzed either enzymatically or chemically to the free volatile compounds during fermentation (Gomez et al. 1994, Francis et al. 1999, Dubourdieu et al. 2006, Fedrizzi et al. 2009). The C_{13}-norisoprenoids (e.g., β-ionone and β-damascenone) fall into this category. Finally, compounds such as amino acids and lipids that are present in grapes are extracted and, as a result of yeast fermentation, are metabolized to yield new compounds that contribute to wine aroma (e.g., alcohols and esters) (Swiegers et al. 2005). Thus, an understanding of the processes that modulate the release of these aroma compounds, or their precursors, could give winemakers an important tool to improve or control wine quality.

doi: 10.5344/ajev.2012.12009

In previous studies evaluating effects of skin contact on volatile composition in white wines, total volatiles generally increased with increased skin contact; C_6-compounds, terpenes, and aromatic alcohols (e.g., 2-phenylethanol) were most influenced by longer skin contact (Ramey et al. 1986, Baumes et al. 1989, Moyano et al. 1994, Cabaroglu et al. 1997, Selli et al. 2003). Increases in levels of these volatile compounds were attributed to extraction of the compounds or their nonvolatile precursors from the skins following maceration of the skins and extraction from cells during alcoholic fermentation. By pressing the skins from Chardonnay grapes at different times during fermentation, the skins also adsorbed some compounds that were initially extracted during maceration (Ferreira et al. 1995). In particular, both the fatty acid precursors and the C_6-alcohol 1-hexanol were sorbed by skins, resulting in overall lower concentrations of C_6-aldehydes and C_6-alcohols in the finished wines following settling and removal of the skins. Therefore, skin contact time may have competing effects on volatile composition depending on relative rates of release from the tissues and physical sorption or binding.

Skin contact during fermentation of red wines also has been generally associated with increased concentrations of many volatile compounds. For Pinot noir, Dornfelder, and Portugieser, extended skin contact resulted in higher concentrations of some alcohols (e.g., 3-methyl-1-butanol and hexanol) (Fischer et al. 2000). Similarly, levels of isoamyl alcohol, 2-methyl-1-butanol, hexanol, and 2-phenylethanol increased in Frontenac juice after two days of skin contact (Mansfield et al. 2011); no concentrations were reported. Concentrations of volatile phenols and the corresponding glycoconjugates that are commonly associated with smoke taint (e.g., guaiacol, 5-methyl guaiacol, and guaiacol glycoconjugates) also increased significantly in wines as skin contact during fermentation increased because of extraction from skins (Ristic et al. 2011). In a study with Pinotage wines, however, prefermentation skin contact had either no effect or decreased acetate ester and ethyl ester concentrations (Marais 2003a). While few studies have focused on Cabernet Sauvignon fermentations or on the time course of volatile production throughout fermentation with and without skins, Schmidt and Noble (1983) used descriptive analysis with trained sensory panelists and observed that overall aroma intensity for Cabernet wines was greater for wines made with longer skin contact. No volatile compounds were measured in the study, however.

Although skin contact is frequently manipulated to optimize color and polyphenol extraction of Cabernet wines, few studies have monitored the effect of skin contact on the composition of Cabernet volatiles throughout fermentation. Accordingly, the goal of the present study was to use a previously developed headspace solid-phase microextraction coupled to gas chromatography-mass spectrometry (HS-SPME GC-MS) method (Canuti et al. 2009) to monitor the effects of skin contact time on the partitioning, release, and formation of volatile compounds during fermentation of Cabernet Sauvignon grapes.

Materials and Methods

Chemicals and reagents. Standards of 2-ethyl-1-hexanol (99+%), (*E*)-2-nonenal (97%), nerol (97+%), β-linalool (97+%), 2-octanone (98%), hexanal (98%), 3-octanol (99%), (*E*)-2-hexenal (98%), ethyl acetate (99%), propyl acetate (99%), ethyl butanoate (99%), isoamyl acetate (99%), hexyl acetate (99%), ethyl hexanoate (99%), ethyl octanoate (99%), and 2-phenylethyl acetate (99%) were purchased from Aldrich (St. Louis, MO). 2-Phenylethanol (99%), β-ionone (95+%), and 1-octanol (99%) were purchased from Sigma (St. Louis, MO). 1-Octen-3-ol (98+%), 1-hexanol (99.9%), and nonanal (95+%) were purchased from Fluka (Sigma-Aldrich, St. Louis, MO). β-Damascenone (1.1 to 1.3% w/w in absolute ethanol) was purchased from SAFC Supply Solution (Sigma-Aldrich). A C_8–C_{20} hydrocarbon mixture, used for determination of Kovats retention indices (KI), was obtained from Fluka (Sigma-Aldrich).

Water was purified through a Milli-Q Water System (Millipore, Billerica, MA) prior to use. Absolute ethanol (200 proof) was purchased from Rossville Gold Shield (Hayward, CA). L-Tartaric acid (99%) was obtained from Aldrich (St. Louis, MO), fructose and diammonium phosphate were from Sigma, and glucose was from Fisher (Fairlawn, NJ).

Fermentations. *Experiment 1.* Cabernet Sauvignon grapes (15 kg) were manually harvested on 2 Sept 2008 from the UC Davis Tyree vineyards (Davis, California) at 21 Brix (pH 3.2). Harvested grapes were transported to the winery and stored overnight at 16°C degrees until winemaking. Grapes were destemmed and crushed with a Zambelli crusher/destemmer (model Cantinetta; Zambelli Enotech, Vicentino, Italy) and the must was manually pressed through a cheesecloth sieve to separate the skins, seeds, and pulp from the juice. The juice yield was ~57% of the destemmed fruit weight. Potassium metabisulfite ($K_2S_2O_5$) was added to bring the must to 50 mg/L total SO_2 prior to beginning the experiment.

Fermentations were carried out in sterilized 1-L glass fermentors (Applikon, Holland). During fermentations, the fermentors were kept in a water bath at 25 ± 0.5°C and were mechanically stirred constantly at 100 rpm, except during SPME sampling.

A total of five treatments were carried out, in duplicate, with the skins (and seeds) removed at various times during fermentations: Skins Long, Skins Short, Juice, Model Juice+Skins, and Model Wine+Skins. For Skins Long, skins (344 g) and juice (456 g) were fermented together for the entire fermentation to simulate the juice/skins ratio of the original grapes. For Skins Short, skins (344 g) and juice (456 g) were fermented together for 72 hr, after which the skins were separated from the liquid and the wine was returned to the fermentation vessel to complete fermentation. For Juice, 800 g of juice only was fermented. For Model Juice+Skins, skins (344 g) were added to a model juice (456 g) prepared with 10.6 g fructose and 10.6 g glucose per 100 g solution and 5.85 g/L tartaric acid (pH adjusted to 3.2 with 6 N NaOH); potassium metabisulfite ($K_2S_2O_5$) was added to bring total SO_2 to 50 mg/L and diammonium phosphate ((NH_4)$_2HPO_4$) was added to a level of 128 mg/L nitrogen to

match that of the Cabernet juice. For Model Wine+Skins, skins (344 g) were added to a model wine (456 g) prepared with 5.85 g/L tartaric acid (pH adjusted to 3.2 with 6 N NaOH) and 12% ethanol (v/v). This last treatment was used to evaluate the influence of ethanol extraction of compounds from the skins, without additional yeast metabolism. Seeds were not separated from skins for any of the treatments and therefore "skins" treatments refer to both skins and seeds throughout this experiment. Model juice and wine were prepared to mimic the sugar, pH, and nitrogen level of the actual juice and wine.

All fermentations were inoculated with the yeast strain *Saccharomyces cerevisiae* Premier Cuvée (Lesaffre Yeast Corporation, Milwaukee, WI) at 0.27 g/L juice. Yeast was first hydrated in five times its weight in Milli-Q water previously warmed to 40°C and after 20 min resting at room temperature the hydrated yeast was added to the juice. Inoculation times were staggered by one hour, so that HS-SPME measurements for equivalent time points could also be staggered by one hour. The fermentations were monitored by weight loss, starting at inoculation and continuing every 12 or 24 hr until the end of fermentation when the weight was stabilized and dryness was confirmed by Clinitest. Model Wine+Skins was discontinued after 120 hr as it appeared that the skins were beginning to ferment and break down.

Experiment 2. Cabernet Sauvignon grapes (45 kg) were harvested on 21 Sept 2010, from the UC Davis Tyree vineyards at 20 Brix (pH 3.6). Harvested grapes were transported to the winery and were used immediately. As in 2008, grapes were destemmed and crushed with a Zambelli crusher/destemmer (model Cantinetta) and the must was manually pressed through a cheesecloth sieve to separate the skins, seeds, and pulp from the juice; skins (including seeds) and juice were weighed separately and juice yield was ~60% of the destemmed fruit weight. Diammonium phosphate ($(NH_4)_2HPO_4$; 50 g/L) was added to the must to bring to 140 g/L, a level similar to that of Experiment 1, potassium metabisulfite ($K_2S_2O_5$) was added to bring the must to 50 mg/L total SO_2, and finally, juice and skins were distributed to the fermentors as described below.

Fermentations were carried out in sterilized 1 L glass fermentors (Applikon). During fermentation, the fermentors were kept in a water bath at 18 ± 0.5°C and were mechanically stirred constantly at 100 rpm, except during SPME sampling.

A total of six treatments were carried out in duplicate, with the skins removed or added at various times during the fermentations: Skins Control, Out 48, Out 84, Juice Control, In 48, and In 84. For treatments where skins were removed, 320 g of skins and 480 g of juice were initially added to each fermentor and the skins remained in the fermentor throughout fermentation (Skins Control) or were removed at 48 hr (Out 48) or 84 hr (Out 84). At the specified time points, skins were removed by gently hand-pressing the fermenting wine through a cheesecloth sieve (weight of skins removed was ~320 g, consistent with the 1.5 juice/skin ratio) and the pressed wine was returned to the fermentor to complete fermentation. For treatments where skins were added to the juice

during fermentation, 480 g of juice was added to each fermentor and either the juice was allowed to complete fermentation (Juice Control) or the skins (~320 g) that were removed from the Out treatments were added to the fermenting juice at 48 hr (In 48) or 84 hr (In 84). As with Experiment 1, skins were not separated from seeds, and therefore references to skins include both skins and seeds throughout Experiment 2.

All fermentations were inoculated with the yeast strain *Saccharomyces cerevisiae bayanus* Prise de Mouse (EC1118; Red Star, Milwaukee, WI) at 0.27 g/L juice. Yeast was first hydrated in five times its weight in Milli-Q water previously warmed to 40°C and was added to the juice after 20 min resting at room temperature. As with Experiment 1, inoculation times for each fermentor were staggered by one hour so that SPME sampling times for equivalent time points could also be staggered. The fermentations were monitored by weight loss, starting at inoculation and continuing every 12 or 24 hr until the end of fermentation when the weight was stabilized and dryness confirmed by Clinitest.

HS-SPME sampling. Volatiles in the headspace of the fermentors were monitored throughout fermentation using the basic procedure of Canuti et al. (2009). A PDMS SPME fiber (polydimethylsiloxane, 100 µm; Supelco, Bellafonte, PA) was used for all analyses. The PDMS fiber resulted in greater extraction of many volatile compounds in Cabernet grapes compared to a mixed phase divinylbenzene/carboxen/PDMS fiber (Canuti et al. 2009). Headspace sampling involved manually inserting a conditioned SPME fiber through a Teflon-coated rubber septum fitted to a sampling port in the lid of the fermentor; the SPME needle was offset ~3 cm from the center of the fermentor to avoid hitting the stirrer. Stirring was stopped during extraction to prevent splashing of the must onto the fiber. The fiber was exposed to the headspace above the liquid for 50 min and then was immediately introduced into the GC-MS inlet. SPME headspace samples were taken starting immediately after inoculation (time 0) and subsequently every 12 to 24 hr until the fermentations were completed and then every 24 to 48 hr for approximately two weeks.

GC-MS analysis. The GC-MS system was an Agilent 7890A GC equipped with an 5975 mass selective detector and Chemstation software (Agilent, Avondale, PA). Instrumental parameters were adapted from Canuti et al. (2009). SPME injections were splitless (straight glass liner, 0.8 mm i.d.) at 240°C for 1 min, during which time thermal desorption of analytes from the fiber occurred. Following SPME desorption, the inlet split flow was turned on (20 mL/min) for the remainder of the GC-MS run and the SPME fiber was conditioned for an additional 9 min before it was removed from the injector. The fiber was immediately placed into the next sample for analysis. There was no carry-over between samples observed with this 10-min total desorption time.

A DB-Wax column (30 m x 0.25 mm i.d., 0.25 µm film thickness) (J&W Scientific, Folsom, CA) was used for all analyses. Helium carrier gas was used with a total flow of 2.33 mL/min. Oven parameters were as follows: initial temperature was 40°C for 4 min, followed by an increase to 80°C at a rate of 2.5°C min, a second increase to 110°C at 5°C min,

and a final increase to 220°C at 10°C min. The oven was then held at 220°C for 5 min before returning to the initial temperature (40°C). The total cycle time, including the oven cool down, was 50 min. The mass detector was operated in scan mode (mass range 50 to 200 *m/z*) and the transfer line to the MS system was maintained at 240°C.

Quantitation and calibration standards. Repeated sampling from the same vessel does not allow for addition of an internal standard to the sample, since the internal standard concentration would change with each sampling. Therefore, a model wine solution was used to prepare standards for calibration curves. Model wine was prepared with Milli-Q water and contained 5.5 g/L tartaric acid and 12% (v/v) ethanol; pH was adjusted to 3.6 with 6 N NaOH. Stock solutions of each standard compound were prepared first in absolute ethanol and then diluted to appropriate levels in the model wine solution (Table 1). For each analyte, a minimum of five concentrations were used to create the calibration curve. Standard solutions were prepared and then a defined volume (800 mL in Experiment 1, 500 mL in Experiment 2) was added to the same type of fermentor used for juice/wine fermentations to replicate the headspace and volume conditions of the actual fermentations as closely as possible. The standard solutions were sampled by HS-SPME for 50 min in exactly the same manner as the fermentation samples. Calibration curves were analyzed in

duplicate at the beginning and end of each experiment and average responses used for calculations.

Calibration standards were also prepared in a model juice solution to evaluate the effects that changes in ethanol concentration during fermentation may have on the quantitation of the volatile aroma compounds. The model juice solution was prepared with Milli-Q water and contained 10.6 g fructose and 10.6 g glucose per 100 g solution, and 5.5 g/L tartaric acid; the pH was adjusted to 3.6 with 6 N NaOH. Standard solutions were prepared and then a total volume of 500 mL was added to the fermentors and HS-SPME analyses were performed as described above. Five concentrations per analyte were used to create the standard curve. Calibration curves were analyzed in duplicate at the beginning and end of each experiment and average responses used for calculations.

GC-MS total ion data were collected for all standards and samples. Peak areas for each compound were obtained using the extracted ions indicated in Table 1; the areas from the standards were plotted against the actual concentration to obtain calibration curves for each compound. The corresponding analyte concentrations in the samples were calculated using the linear regression equations.

Compound identification. Compounds were identified by comparison of mass spectra to those in the ChemStation NIST Spectral Library, by comparison of calculated retention indices

Table 1 Volatile compounds measured during Cabernet Sauvignon fermentations.

Compound	Calculated KI (literature)[a]	*m/z*[b]	Linear range (µg/L)	S/N for lowest standard	r
Ethyl acetate	–[c]	61	50–150,000	22	>0.99
Propyl acetate	–[c]	61	250–1000	143	>0.99
Ethyl butanoate	1031 (1028)	71	5–1000	20	>0.99
Hexanal	1073 (1067)	56	20–20,000	135	>0.99
Isoamyl acetate	1115 (1118)	70	500–150,000	590	>0.99
(*E*)-2-Hexenal	1204 (1212)	55	20–10,000	13	>0.99
Ethyl hexanoate	1225 (1224)	88	1–1500	60	>0.99
Hexyl acetate	1264 (1264)	56	1–1500	44	>0.99
2-Octanone	1275 (1283)	58	0.01–100	47	>0.99
1-Hexanol	1355 (1345)	56	50–10,000	27	>0.99
Nonanal	1384 (1392)	57	0.01–100	58	>0.99
3-Octanol	1396 (1395)	59	1–1000	10	>0.99
Ethyl octanoate	1432 (1435)	88	1–1500	169	>0.99
1-Octen-3-ol	1452 (1465)	57	5–50	22	>0.99
2-Ethyl-1-hexanol	1490 (1494)	57	10–1000	141	>0.99
(*E*)-2-Nonenal	1529 (1532)	70	0.5–1000	23	>0.99
β-Linalool	1552 (1545)	71	1–100	10	>0.99
1-Octanol	1563 (1565)	56	1–100	6	>0.99
Nerol	1803 (1805)	69	0.1–1000	38	>0.99
2-Phenylethyl acetate	1815 (1803)	104	5–1000	462	>0.99
β-Damascenone	1824 (1841)	69	0.24–62[d] 0.5–10[e]	25 60	>0.99
2-Phenylethanol	1918 (1925)	91	500–7500	250	>0.99
β-Ionone	1951 (1956)	177	0.1–50	200	>0.99

[a]KI: Kovats index. Literature sources: www.odour.org.uk, www.flavornet.org, www.pherobase.com, Jennings and Shibamoto (1980).
[b]Ion fragment, extracted from total ion chromatogram, that was used for quantitation.
[c]Ethyl acetate retention time = 2.6 min; propyl acetate retention time = 3.9 min. Confirmed with authentic standards.
[d]Calibration range used for Experiment 1.
[e]Calibration range used for Experiment 2.

to those available from the literature (Jennings and Shibamoto 1980), and by injection of authentic standards.

Statistical analysis. Means, standard deviations, relative standard deviations (RSD), and linear regressions were calculated in Excel (Microsoft, Redmond, WA). One-way ANOVA was performed to evaluate significant differences (significance levels $p < 0.05$) using Statistica software (ver. 7.0; StatSoft, Tulsa, OK) or R system, an open-source data analysis program (www.r-project.org).

Results and Discussion

Fermentation. In both experiments, the fermentation profiles (as monitored by weight loss) were virtually identical for all treatments and all duplicate fermentations. Example fermentation profiles for the control fermentations carried out with and without skins in Experiment 2 (i.e., Skins Control and Juice Control, respectively) are shown in Figure 1. Fermentation profiles were similar for Experiments 1 and 2, but overall the fermentations were faster in Experiment 1 (profile not shown), consistent with the slightly higher fermentation temperature in Experiment 1. The initial lag time was ≤24 hr for both experiments; after initiation, the exponential phase for the fermentations was ~2 days for Experiment 1 and ~4 days for Experiment 2. Fermentations were complete and the weight stabilized after 3 to 4 days for Experiment 1 and after 5 to 6 days for Experiment 2. The mean pH of the wines was 3.2 and 3.6 ± 0.2 and ethanol levels were 10.8 ± 0.5% (v/v) and 12.0 ± 0.3% (v/v) for Experiments 1 and 2, respectively.

Standard calibration curves. During fermentation, sugar and ethanol concentrations change, which can influence the partitioning of volatiles into the headspace (Vianna and Ebeler 2001, Robinson et al. 2009). Therefore, calibration curves were prepared using two model solutions, one with 12% ethanol (model wine) and the other with sugar (glucose

and fructose) but no ethanol (model juice). No significant differences in results were observed between the model juice and model wine standards when used with the HS-SPME procedure described (data not shown), perhaps due to the relatively long (50 min) sampling time which allows the volatile compounds to reach an equilibrium between the solution, vapor, and SPME fiber phases. Under these conditions, HS-SPME measurements are not as influenced by the solution properties as shorter sampling times would be (Roberts et al. 2000, Jung and Ebeler 2003, Lloyd et al. 2011). Other authors (Conner et al. 1998, Luan et al. 2000) have also observed that for solutions of less than 17% (v/v) ethanol, there were no significant differences in headspace concentration of esters as measured by a static headspace procedure. Further, at a given time point all treatments will have a similar ethanol concentration and matrix composition, and if ethanol does influence the measured concentration, then the relative effects will be similar across treatments and relative differences among treatments can still be compared. Here, all concentrations of all volatile compounds are reported using the calibration curves performed using the model wine solutions (Table 1).

Analysis of volatiles during fermentation. For all of the analytes measured, ANOVA showed no significant differences ($p < 0.05$) between each of the two replicate fermentations for each fermentation treatment (data not shown). Therefore, data from duplicate fermentors were combined and average values are presented.

Among all of the compounds for which standards and calibration curves were prepared, several compounds were not detected at all or were present at concentrations below their quantitation limits (lowest concentration of calibration curve): propyl acetate, 1-octen-3-ol, 2-octanone, 3-octanol, (E)-2-nonenal, hexanal, 2-ethyl-1-hexanol, β-linalool, and nerol. These compounds have previously been reported to occur in low concentrations in Cabernet Sauvignon wines (0 to 16 µg/L) (Pineiro et al. 2006, Canuti et al. 2009, Jiang and Zhang 2010). 3-Isobutyl-2-methoxypyrazine (IBMP) is also often present at very low levels (ng/L) in Cabernet grapes and wines and contributes a distinctive bell pepper aroma to this variety. Detection and quantitation of IBMP at these trace levels typically requires GC-MS analysis using selected ion monitoring (Chapman et al. 2004). Here, we chose to profile volatiles using the total ion chromatogram in order to simultaneously measure as many compounds as possible throughout the fermentations. Future studies are needed to focus on changes in concentrations of these trace compounds as a function of skin contact during fermentation.

Experiment 1. In the initial experiment, we hypothesized that compounds extracted from grape skins would be present in higher concentrations in treatments with skin contact throughout all, or part, of the fermentation, while fermentation-derived alcohols and esters would be less dependent on skin contact/extraction for production of volatiles. Overall, there were several general patterns of volatile concentration profiles during the time course of the fermentations, with the profiles dependent on the compound being measured (Figure 2). Measured levels and means comparisons for each of the
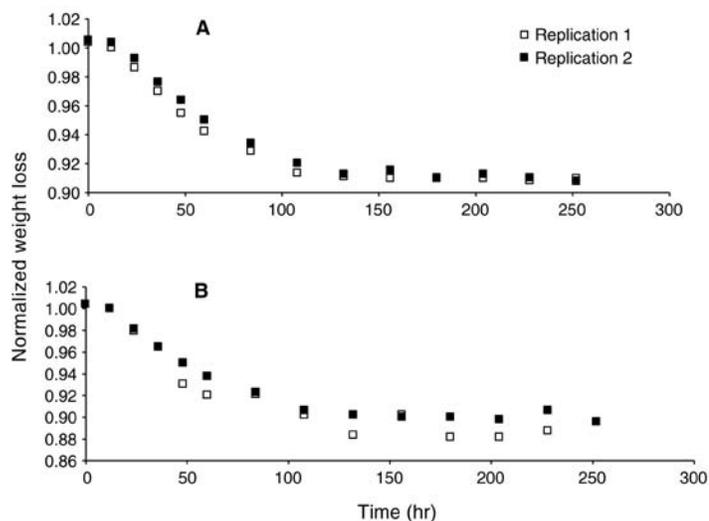

Figure 1 Fermentation curves for Experiment 2. Relative weight loss for duplicate fermentations of (**A**) Skins Control and (**B**) Juice Control treatments.

analyte concentrations at the initial time, at the time when maximum concentrations were reached, and at the end of the fermentations are provided in Supplemental Tables.

For the C_6-compounds, 2-hexenal and 3-hexen-1-ol, the measured concentrations were highest when both skins and juice were present for at least 72 hr of the fermentation (Skins Long and Skins Short) (Figure 2A, data shown for 2-hexenal only). This was then followed by a rapid decline in concentration as the compounds volatilized or further reacted (chemically or enzymatically) to form new compounds. 1-Hexanol levels increased to ~1500 µg/L during the first 24 hr of fermentation and then decreased to concentrations of ~800 µg/L for Skins Long, Skins Short, and Juice (see also Supplemental Tables).

The grape-derived C_{13}-norisoprenoid, β-ionone, demonstrated a different pattern of release and extraction (Figure 2B). Measured concentrations increased during the first 24 hr of fermentation for all but Model Wine+Skins; following this initial increase, concentrations decreased rapidly over the next 24 hr of fermentation. Compared to the C_6-compounds, there was a slower extraction and release of β-ionone from skins when ethanol concentrations were low at the beginning of the fermentation. There was little difference in the concentration of β-ionone in any of the samples fermented with actual grape juice, regardless of skin contact time, while Model Juice+Skins had lower overall β-ionone levels, indicating that the juice contributed to the total levels of β-ionone during fermentation and in the final wine. For skins extracted with
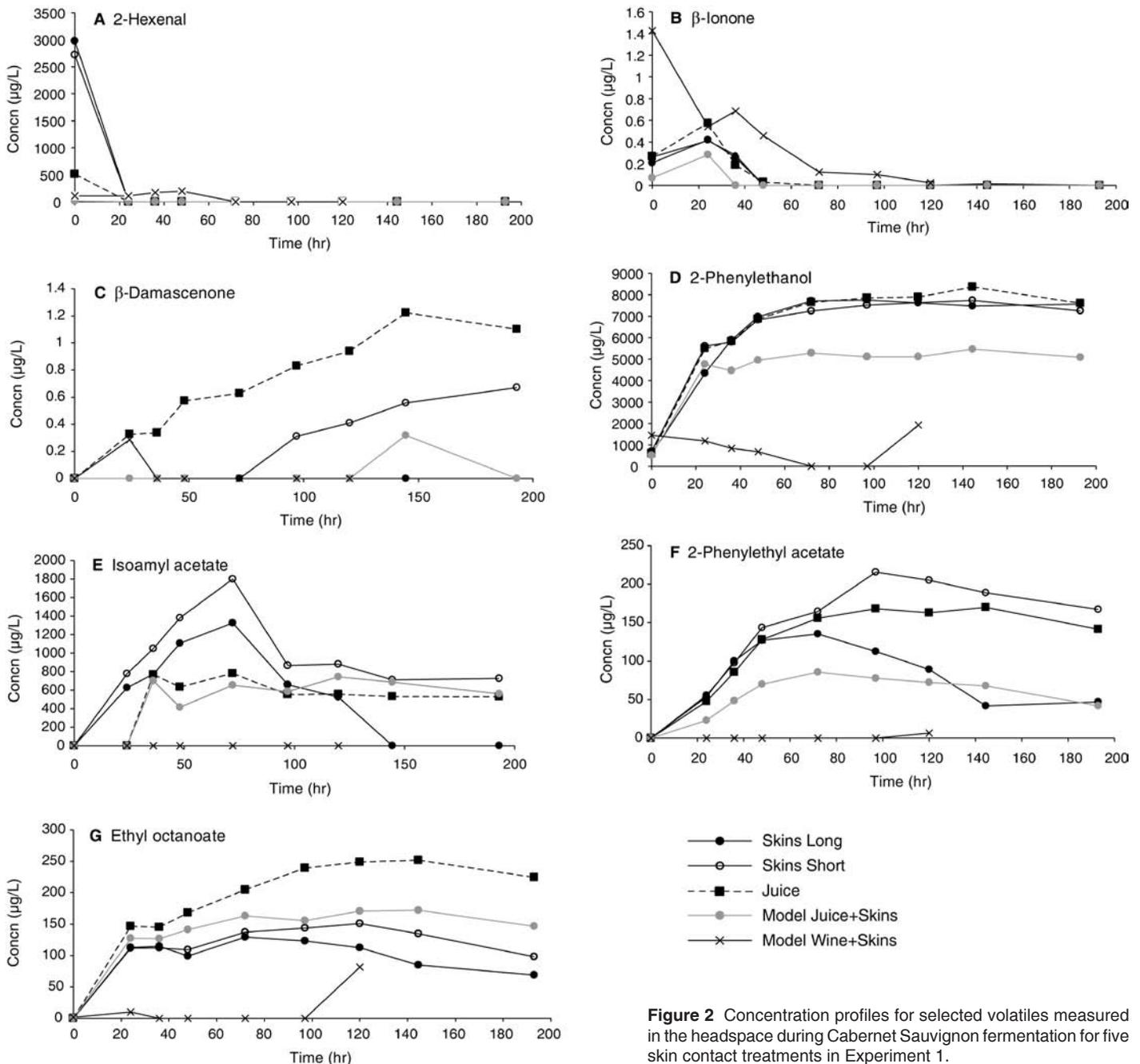

Figure 2 Concentration profiles for selected volatiles measured in the headspace during Cabernet Sauvignon fermentation for five skin contact treatments in Experiment 1.

the model wine (containing 12% ethanol), there was a high level of extraction initially, but then the β-ionone decreased over approximately three days.

β-Damascenone, another grape-derived C_{13}-norisoprenoid, exhibited a very different pattern of extraction and release than β-ionone (Figure 2C). Concentrations in the Juice treatment increased continuously throughout fermentation, consistent with previous work showing that β-damascenone concentrations increase during fermentation via transformation of the ketone precursors by yeast (Lloyd et al. 2011). For Skins Short, the levels of β-damascenone remained low until the skins were removed at 72 hr, and then the concentration in the wine increased at a rate similar to that observed for the Juice treatment. In contrast, when skins were present throughout the entire fermentation (Skins Long and Model Juice+Skins), β-damascenone remained below and/or near the limit of quantitation throughout the fermentation. Small amounts of β-damascenone were extracted by ethanol in Model Wine+Skins; however, concentrations decreased to below the limit of quantitation within 36 hr. These results present an unexpected effect of skins on β-damascenone extraction and release. While β-damascenone can be extracted and released from both skins and juice, it also appears that skins can bind to this compound, decreasing its volatility, or, alternatively, that the presence of the skins affects release and hydrolysis of the nonvolatile precursors during fermentation.

Fermentation-derived alcohols, including 2-phenylethanol (Figure 2D) and isoamyl alcohol, increased in concentration during fermentation for all treatments. There was little effect of skin contact and all grape juice fermentations with or without skins had similar levels of these alcohols. For 2-phenylethanol (but not isoamyl alcohol), extraction from the grape skins by ethanol in the model wine solution occurred initially, but levels remained low and relatively constant. Low levels of 2-phenylethanol have been reported in Cabernet grapes (Canuti et al. 2009).

Acetate esters (isoamyl acetate and 2-phenylethyl acetate) were not present initially in the samples, and concentrations increased throughout the fermentations, reaching a maximum at approximately the point where fermentations were complete (~72 hr). While concentrations of both of these esters followed similar patterns during fermentation, some differences were observed. Isoamyl acetate decreased rapidly after completion of the fermentation when skins were present (Skins Long and Skins Short), and concentrations for these treatments reached higher maximum levels than in Juice and Model Juice+Skins (Figure 2E). On the other hand, concentrations of 2-phenylethyl acetate did not decrease as much after reaching maximum levels, with the exception of the Skins Long treatment (Figure 2F). The Juice and Skins Short treatments had the highest levels of 2-phenylethyl acetate and the acetate esters were not extracted from the Model Wine+Skins treatment containing 12% ethanol. These results are consistent with the known route of acetate ester formation by yeast acetyltransferase enzymes during fermentation (Swiegers et al. 2005).

Several ethyl esters were formed during the fermentations, including ethyl butanoate, ethyl hexanoate, and ethyl octanoate. All followed similar profiles during the fermentations, and for simplicity, only data from ethyl octanoate are shown (Figure 2G). These esters were not present initially in measurable amounts; concentrations began to increase in the first 24 hr and continued to increase until the midpoint of the exponential phase of the fermentation (~72 hr); concentrations remained constant or decreased slightly after reaching maximum levels. These esters were not present in Model Wine+Skins since they are formed during fermentation. Overall, concentrations were highest in the Juice treatment without skins and lowest when skins were present throughout fermentation (Skins Long). Elsewhere, ethyl ester concentrations were lower in Pinotage wines fermented after extended prefermentation skin contact at 15°C compared to no prefermentation skin contact; however, no quantitative data were presented (Marais 2003a). In the current study, the presence of skins had less effect on overall levels of shorter chain ethyl esters (ethyl butanoate and ethyl hexanoate) throughout the fermentation than on longer chain esters.

Based on these patterns, the presence of grape skins appears to influence extraction, release, and formation of volatiles during fermentation. However, the hypothesis that extraction of grape-derived compounds is directly related to skin contact time was not confirmed for all compounds and for some compounds the concentration in the headspace was decreased by the presence of skins. Therefore, Experiment 2 explored potential mechanisms for the observed effects of skin contact on concentrations of volatiles during fermentation, particularly concentrations of β-damascenone. It is possible that skins, in addition to providing a rich source of grape-derived volatiles, could bind some compounds, decreasing their concentrations and volatility. Therefore, in Experiment 2 skins were added to, as well as removed from, the samples during the time course of the fermentations.

Experiment 2. At the beginning of the fermentations, only two compounds, (*E*)-2-hexenal and 1-hexanol, were quantifiable at concentrations above their detection limits (Table 2). Similar to Experiment 1, 2-hexenal was initially present at high concentrations (higher in the Cabernet musts when skins were present compared to juice) and then decreased to below measurable levels by the end of fermentation. On the other hand, 1-hexanol generally increased during the first 24 to 48 hr of fermentation; after reaching a maximum, levels decreased slightly but then remained relatively constant (Table 2, Figure 3A). In general, the C_6-aldehydes and C_6-alcohols are formed from enzymatic and chemical oxidation of fatty acid precursors extracted from grape skins. The rapid extraction and subsequent degradation or loss of 2-hexenal, combined with increasing levels of 1-hexanol, are consistent with (*E*)-2-hexenal being reduced to 1-hexanol within 48 hr under wine fermentation conditions, as previously reported (Joslin and Ough 1978). In addition, pressing the skins off after 48 hr (Out 48) resulted in a decrease in final 1-hexanol levels compared to the Skins Control sample where skins were present throughout fermentation (Table 2; Figure 3A); this is possibly a result of sorption of either the fatty acid precursors or 1-hexanol to the skins, as previously

Table 2 Mean concentrations of volatile compounds determined at the beginning (I: initial) and at the end (F: final) of fermentations for Experiment 2. Concentrations reported as μg/L ± standard deviation unless otherwise noted.

Compound[a]	Juice Control		In 48		In 84		Skins Control		Out 48		Out 84	
	I	F	I	F	I	F	I	F	I	F	I	F
Ethyl acetate[b]	nd[c]	29 ± 3 a[c]	nd	56 ± 3 b	nd	58.4 ± 2.3 b	nd	91.6 ± 1.1 c	nd	69 ± 3 d	nd	93.0 ± 0.8 c
Ethyl butanoate	nd	260 ± 6 a	nd	254 ± 30 a	nd	257 ± 8 a	nd	420 ± 30 b	nd	226 ± 7 a	nd	430 ± 55 b
Isoamyl acetate	nd	1966 ± 112 a	nd	<LOQ	nd	723 ± 64 b	nd	1406 ± 124 c	nd	5781 ± 507 d	nd	9191 ± 732 e
(E)-2-Hexenal	<LOQ[c]	nd	<LOQ	nd	38.04 ± 0.03 a	nd	22.43 ± 0.03 a	nd	31.4 ± 1.3 b	nd	39 ± 5 b	nd
Ethyl hexanoate	nd	679 ± 106 a	nd	657 ± 72 a	nd	696 ± 101 a	nd	1255 ± 129 b	nd	782.7 ± 0.3 a	nd	1211 ± 166 b
Hexyl acetate	nd	96 ± 15 a	nd	9.3 ± 0.8 b	nd	13.6 ± 0.4 b	nd	17.6 ± 2.1 b	nd	312 ± 38 c	nd	255 ± 35 c
1-Hexanol	86 ± 13 a	1787 ± 6 b	70 ± 11 a	3472 ± 252 c	76 ± 9 a	3304 ± 67 c	104.91 ± 0.03 a	2902 ± 115 c	103 ± 12 a	1940 ± 61 b	116 ± 12 a	2452 ± 168 d
Ethyl octanoate	nd	818 ± 133 a	nd	750 ± 81 a	nd	749 ± 115 a	nd	1355 ± 143 b	nd	1121 ± 106 b	nd	1476 ± 142 b
1-Octanol	nd	11.1 ± 1.2 a	nd	17.2 ± 2.2 b	nd	18.9 ± 1.8 b	nd	24 ± 4 c	nd	9.7 ± 0.4 a	nd	16.1 ± 2.1 b
2-Phenylethyl acetate	nd	484 ± 12 a	nd	19.1 ± 1.6 b	nd	18.08 ± 1.01 b	nd	20.7 ± 1.9 b	nd	840 ± 84 c	nd	552 ± 68 d
β-Damascenone	nd	4.3 ± 0.3 a	nd	0.91 ± 0.10 b	nd	0.88 ± 0.15 b	nd	0.735 ± 0.007 b	nd	1.84 ± 0.18 c	nd	1.43 ± 0.12 b
2-Phenylethanol[b]	nd	5.39 ± 0.11 a	nd	4.4 ± 0.4 a	nd	4.78 ± 0.19 a	nd	4.3 ± 0.6 a	nd	4.8 ± 0.4 a	nd	3.91 ± 0.03 a
β-Ionone	nd	0.446 ± 0.02 a	nd	0.445 ± 0.02 a	nd	0.50 ± 0.07 a	nd	0.470 ± 0.022 a	nd	0.410 ± 0.015 a	nd	0.448 ± 0.005 a

[a]Retention indices and mass spectra for all compounds confirmed with authentic standards as given in Table 1, except for ethyl acetate and propyl acetate where KI were not calculated, but retention times and mass spectra were confirmed with authentic standards.
[b]Concentration reported in mg/L.
[c]nd: no peak detected. <LOQ: below limit of quantitation, lowest standard in calibration curve. Initial and final concentrations with different letters (a, b, c, d) are significantly different ($p < 0.05$).

observed (Ferreira et al. 1995). A longer skin contact for 84 hr of fermentation (Out 84) did not have as great of an effect on decreasing hexanol levels at the end of the fermentation, although all treatments with skins present at the beginning of fermentation had lower 1-hexanol levels than when skins were added to the fermentations at 48 and 84 hr (In 48 and In 84). Adding skins to juice at 48 and 84 hr resulted in the highest 1-hexanol concentrations at the end of the fermentation, possibly due to extraction of fatty acids as ethanol levels increased and cells were disrupted during fermentation. Overall, these results demonstrate equilibrium among extraction, formation, and sorption of 1-hexanol to skins and the relative importance of each of these competing mechanisms is dependent on the timing and duration of skin contact time. 1-Hexanol has an herbaceous aroma (Falqué and Fernández 1996), and the concentrations observed in this study (2 to 3.5 mg/L) were similar to those found in young red wines (Ferreira et al. 2000).

The grape-derived C_{13}-norisoprenoid β-ionone was present in similar concentrations at the end of all fermentations performed with or without skins (Table 2; Figure 3B). During fermentation, β-ionone concentrations reached a maximum ~48 hr after the fermentation was initiated. This profile is consistent overall with Experiment 1, although concentrations remained higher in Experiment 2. β-Ionone is formed from photochemical oxidation and/or enzymatic cleavage of β-carotene, which is generally at highest concentrations in the skins (Enzell 1985, Kotseridis et al. 1999, Mendes-Pinto 2009). Here, both skins and pulp/juice appeared to be a source of β-ionone during fermentation (Figure 2B, 3B). β-Ionone is also typically present as a glycoside precursor in grape tissues and the presence of free β-ionone, as was measured here, requires enzymatic or chemical hydrolysis of the glycosides to yield the free aglycone. Results from Experiment 1 indicated that β-ionone can be rapidly extracted by ethanol from skins, but it is also rapidly degraded. Therefore, final levels of this compound at the end of fermentation are the summation of extraction, hydrolysis, degradation, and volatilization. β-Ionone is described as having the aroma of violets and has been found in many grapes (López et al. 1999) and wines, including California Cabernet Sauvignon wines (Kotseridis et al. 1999). Levels of β-ionone at the end of the fermentation were low but higher than those found in a set of young red Spanish wines, including Cabernet Sauvignon (Ferreira et al. 2000).

As in Experiment 1, β-damascenone levels were significantly influenced by addition and removal of skins (Table 2, Figure 3C). When skins were added to the juice at 48 or 84 hr (In 48 and In 84), β-damascenone decreased significantly. Conversely, when skins were removed at either 48 or 84 hr (Out 48 and Out 84), β-damascenone increased at a rate similar to that observed for the Juice Control. Like β-ionone, β-damascenone is a C_{13}-norisoprenoid formed from enzymatic or chemical cleavage of carotenoid precursors and it exists as a nonvolatile precursor in grape skins and pulp (Sefton et al. 2011). As has been observed, β-damascenone levels increased as a result of yeast fermentation (Lloyd et al. 2011); however,

our results also indicate that β-damascenone or its precursors may be sorbed by the skins or, alternatively, components in the skins may inhibit extraction and formation of the free aglycone during fermentation. This effect of skin contact for β-damascenone, where similar effects were not observed with the related compound β-ionone, points to a potentially highly specific binding or inhibition mechanism; however, further studies are needed to fully distinguish between these two possibilities. β-Damascenone is a potent odorant on its own and can enhance the aroma of other compounds (Pineau et al. 2007); it contributes to the aroma of many wine varieties including Cabernet Sauvignon (Kotseridis et al. 1999, López et al. 1999). Concentrations of β-damascenone observed in

this study were similar to those previously reported (Sefton et al. 2011).

Profiles for concentrations of fermentation-derived alcohols and esters were generally similar in Experiment 2 and Experiment 1 (Table 2, Figure 3D-H, for simplicity, only profiles for representative compounds are shown; profiles for additional compounds are provided in Supplemental Tables). The presence of skins had the greatest effect on 2-phenylethyl acetate concentrations (Figure 3F), and skins appeared to bind or inhibit formation of this compound, as in Experiment 1. Formation of fatty acid ethyl esters was not impacted by addition or removal of skins (Figure 3G); however, overall concentrations of these esters reached the highest levels
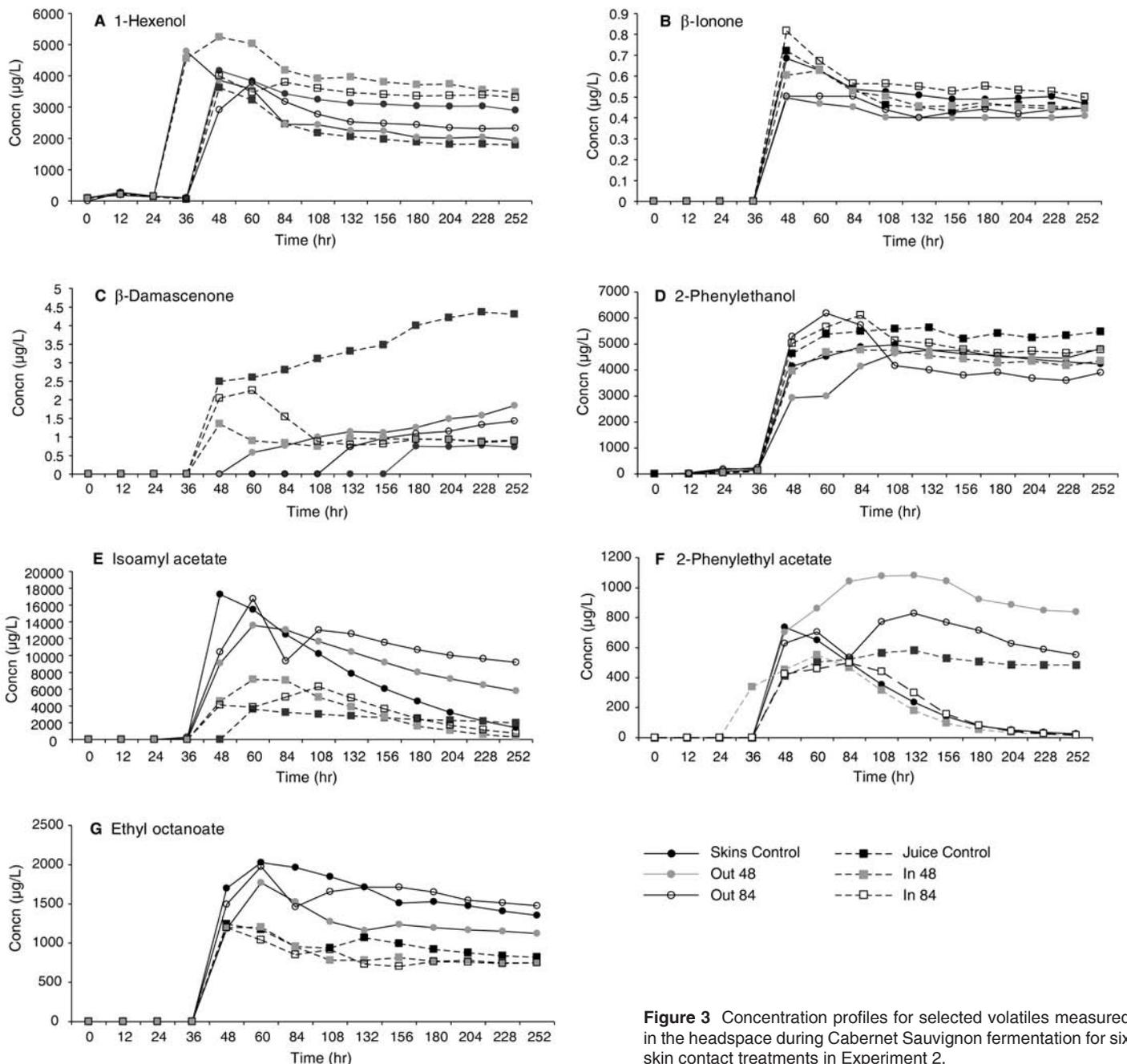

Figure 3 Concentration profiles for selected volatiles measured in the headspace during Cabernet Sauvignon fermentation for six skin contact treatments in Experiment 2.

and remained so when skins were present for the first 48 hr of fermentation (Skins Control, Out 48, and Out 84). This is consistent with release of amino acids (which can serve as precursors to the esters) into the must during the early part of the fermentation as reported elsewhere (Hernandez Orte et al. 1998). The effects of skin contact on increasing ethyl ester concentrations were not consistent between the two experiments, however, and may have been due to differences between the two experiments in temperature (25°C in Experiment 1 and 18°C in Experiment 2) or in juice nutrients and yeast strains. Temperature, yeast strain, and juice nutrients can significantly impact formation of ethyl esters during fermentation (Ramey et al. 1986, Lee et al. 2004, Miller et al. 2007). Premier Cuvee and Prise de Mousse are from the same progenitor strain but have been grown separately. They are often used interchangeably, but they may exhibit some differences in metabolism (Prior et al. 2000, Dunn et al. 2005). Yeast biomass can also influence ester concentrations (Lubbers et al. 1994, Lee et al. 2004, Chalier et al. 2007). Although similar inoculation rates were used for Experiments 1 and 2, the time to complete fermentation was slightly different and may be due to differences in biomass. We did not measure yeast biomass during the fermentations in order to avoid repeated opening of the fermentors; however, this effect should be further evaluated in combination with skin contact time. Tannins and polyphenols, present in skins and extracted during fermentation, can interact with ethyl esters and some other volatile compounds to decrease their volatility (Aronson and Ebeler 2004, Rocha et al. 2007). However, we did not observe evidence for this volatile/tannin interaction here with the ethyl esters, possibly because of the long HS-SPME extraction times, which would disrupt the equilibrium of the noncovalent interactions (Robinson et al. 2009). The fermentation alcohols and esters measured in this study contribute fruity and floral aromas to most wines, including those made from Cabernet grapes (Kotseridis and Baumes 2000, Ferreira et al. 2000, Gürbüz et al. 2006).

These experiments were conducted using bench-scale 1 L fermentors with continuous stirring. In pilot and industrial-scale fermentations, juice and skin contact is usually ensured by periodic pump-overs (juice from bottom of tank is pumped to the top of the tank and sprayed over the skin cap) or punch-downs (skin cap is forcibly pushed to the bottom of the tank and allowed to mix with the juice as it slowly rises to the surface). In Pinotage fermentations with pump-over or punch-down treatments, no significant effects on acetate and ethyl esters concentrations were observed (Marais 2003b). Further study is needed to evaluate volatile composition as a function of different fermentation scales and to evaluate different methods of ensuring juice and skin contact during fermentation.

In order to add or remove skins during the fermentations, the fermentors were opened to ambient air, and to remove skins the fermenting must was filtered. These processes will result in incorporation of more oxygen into these treatments. Although there did not appear to be effects of oxygen incorporation in these treatments (i.e., significantly higher levels of aldehydes or alcohols), further study is needed to evaluate combined effects of oxygen addition and skin contact time on volatile concentrations during fermentations.

Conclusions

Results demonstrated that the duration and timing of skin contact during fermentation can have a significant effect on volatile composition of Cabernet Sauvignon wine. In some cases, the presence of skins tended to result in higher maximum concentrations of some volatile compounds after the completion of fermentation, as was observed for many, but not all, fermentation-derived alcohols and esters. However, by monitoring the volatile composition throughout the fermentation, we observed that skins had a significant effect on sorbing or delaying the release and/or formation of other compounds such as β-damascenone. Overall, there is a complex interplay among extraction, formation, volatilization, and sorption of volatiles during grape fermentations and more studies are needed to fully understand how these variables influence the final wine composition. The effects that the observed changes in chemical composition may have on sensory properties of the final wines are unknown; therefore, future studies linking the observed changes to sensory properties are necessary to provide winemakers with improved tools for optimizing wine composition and aroma during fermentation.

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