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# Grape Contribution to Wine Aroma: Production of Hexyl Acetate, Octyl Acetate, and Benzyl Acetate during Yeast Fermentation Is Dependent upon Precursors in the Must

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**ABSTRACT:** Wine is a complex consumer product produced predominately by the action of yeast upon grape juice musts. Model must systems have proven ideal for studies of the effects of fermentation conditions on the production of certain wine volatiles. To identify grape-derived precursors to acetate esters, model fermentation systems were developed by spiking precursors into model must at different concentrations. Solid-phase microextraction−gas chromatgraphy mass spectrometry analysis of the fermented wines showed that a variety of grape-derived aliphatic alcohols and aldehydes are precursors to acetate esters. The C6 compounds hexan-1-ol, hexenal, (*E*)-2-hexen-1-ol, and (*E*)-2-hexenal are all precursors to hexyl acetate, and octanol and benzyl alcohol are precursors to octyl acetate and benzyl acetate, respectively. In these cases, the postfermentation concentration of an acetate ester increased proportionally with the prefermentation concentration of the respective precursor in the model must. Determining viticultural or winemaking methods to alter the prefermentation concentration of precursor compounds or change the precursor-to-acetate ester ratio will have implications upon the final flavor and aroma of wines.

**KEYWORDS:** *fermentation, ester, hexanol, precursor, wine, grape, SPME-GCMS, hexyl acetate*

## INTRODUCTION

Wine, as produced by the action of yeast on grape juice musts, stands as a beverage of high commercial importance and consumer regard. Much of the appeal of wine can be attributed to the varied nature of the product. Wines can be distinguished by grape variety (for reviews, see refs 1 and 2), geographic location of vineyards,[3] and variations in the same vineyard.[4] Different viticultural practices as well as winemaking and aging techniques can vary greatly the sensory attributes of wines (e.g., refs 5−7). These variables can alter the final concentration of volatile compounds in wines, and these compounds play a vital role in the perception of flavor and aroma by the consumer. Understanding the nature and origins of wine volatile compounds provide the potential to manipulate grape-growing and wine-making practices toward producing wines that offer flavor and aroma characteristics desired by targeted consumer groups.

The role of the grape in providing volatile aroma compounds to wines has received much attention (see refs 1, 2, 8, and 9). Much of this research has focused upon varietal impact compounds. These are compounds that are specific to a grape variety (or a small number of varieties) and contribute strongly to the characteristic aromas of wines made from those varieties. Such compounds include terpenoids, norisoprenoids, volatile thiols, and methoxypyrazines. Many of these varietal impact compounds will be present in grapes as both free and "bound" forms, and the composition and concentrations of these compounds will vary depending on the variety.[10−12] Grapes also provide neutral compounds such as C6 alcohols and aldehydes, which can impact upon the aroma of wines and are common to all varieties.

The contributions of grapes to the production of fermentation-derived esters, such as ethyl and acetate esters, are less well understood. Fang and Qian[13] showed that grapes of different maturities produced varying concentrations of volatile esters using controlled fermentation conditions. Moio et al.[14] also showed that a number of esters contributed significantly to the varietal aroma of Pinot Noir. Other reports have also implicated esters in varietal aroma of young red wines.[15,16] Additionally, recent work by Pineau et al.[17] disclosed that small variations in wine ester concentration can affect wine aroma. Given that esters are one of the most abundant classes of volatile compounds found in wine,[18] understanding the factors involved in their production is of critical importance to the potential for manipulating wine aroma.

Model grape juice media (MGJM) have become a popular tool for gauging volatile compound production during alcoholic fermentation. MGJM generally contain a simple mix of sugars and nutrients sufficient to sustain the yeast during fermentation. Through using MGJM, fermentation can be carefully controlled so the effects of changing one or more variables can be observed. Several studies have employed controlled MGJM fermentations to monitor changes in volatile compound production during and after fermentation while altering

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variables such as amino acid profile,[19] carbon and nitrogen content,[20,21] fermentation temperature,[22,23] and yeast strain,[21,24] to name a few.

A recent report by Keyzers and Boss,[25] involving the spiking of varying proportions of Riesling or Cabernet Sauvignon grape juice into MGJM, showed the grape dependence of a number of fermentation-derived esters. That is, the production of some esters increased during fermentation as the proportion of grape juice to MGJM increased. A particularly dramatic example was hexyl acetate. The postfermentation concentrations of this ester were 30- and 140-fold higher in 100% Riesling juice and 100% Cabernet Sauvignon juice ferments, respectively, when compared to MGJM ferments (0% grape juice). Hexyl acetate is not present to any significant extent in grape juice musts;[26,27] it is a product of yeast fermentation. Also, hexyl acetate has been shown to be associated with red berry aroma,[28] a usually pleasant aroma descriptor in red wines. This paper describes further experiments to gain an understanding of the factors involved in the production of hexyl acetate during yeast fermentation and identifies grape-derived components that influence the concentration of this ester in wines.

## MATERIALS AND METHODS

**Spiking Compounds.** All precursor compounds used for fermentation spiking were purchased from Sigma-Aldrich (Castle Hill, Sydney, Australia) and used without further preparation.

**Grapes.** Berries from the cultivar Riesling were machine harvested from a commercial vineyard in Eden Valley, South Australia, in the 2006 vintage. Bunches were destemmed and pressed, and the free-run juice settled at 4 °C for 4 days after the addition of $SO_2$ (50 ppm). Aliquots were flash frozen in cut-down wine cask liners using liquid $N_2$ and stored at −80 °C until required. Bunches of Cabernet Sauvignon berries, grown in a commercial vineyard in Waikerie, South Australia, were collected by hand in the 2008 vintage. Berries were destemmed by hand and flash frozen in liquid $N_2$ before storage at −80 °C until needed. When required, Cabernet Sauvignon berries were ground in a blender under liquid $N_2$ after which $SO_2$ (50 ppm) was added. The resulting powder was allowed to thaw at 4 °C overnight after which it was centrifuged (4000 rpm for 15 min) to remove pomace (seeds, skins, pulp, etc.), producing clarified juice for determination of approximate C6 compounds concentrations.

**MGJM.** MGJM was prepared based on the protocol reported by Keyzers and Boss[25] with slight modifications. D-Glucose (120 g), D-fructose (120 g), 5 g of D/L-malic acid, 5 g of tartaric acid, 0.2 g of citric acid, 15 mg of ergosterol, 5 mg of sodium oleate, 2 mg of nicotinic acid, 1.7 g of yeast nitrogen base (YNB) without ammonium sulfate (1000 mg/L $KH_2PO_4$, 2 mg/L *myo*-inositol, 0.04 mg/L $CuSO_4$, 500 mg/L $MgSO_4$, 0.4 mg/L niacin, 0.1 mg/L KI, 100 mg/L NaCl, 0.2 mg/L *para*-aminobenzoic acid, 0.2 mg/L $FeCl_3$, 100 mg/L $CaCl_2$, 0.4 mg/L pyridoxine, 0.4 mg/L $MnSO_4$, 0.002 mg/L biotin, 0.2 mg/L riboflavin, 0.2 mg/L $Na_2MoO_4$, 0.4 mg/L calcium pantothenate, 0.4 mg/L thiamine, 0.4 mg/L $ZnSO_4$, 0.002 mg/L folic acid, 0.5 mg/L $H_3BO_3$; MP Biomedicals, Santa Ana, CA), 8 g of Synthetic Complete (Hopkins) amino acid supplement mixture (684.8 mg/L L-leucine, 342.4 mg/L of the other 19 standard amino acids, 342.4 mg/L *myo*-inositol, 342.4 mg/L uracil, 84 mg/L adenine, and 34.4 mg/L *para*-aminobenzoic acid), 0.3 g $NH_4Cl$, and 0.5 mL of Tween 80 were dissolved in 1 L of water. The pH of the resulting medium was corrected to 3.20 by the addition of KOH. The final yeast assimilable nitrogen (YAN) of the MGJM was calculated at 790 ± 12 mg N/L. This was determined using an *o*-phthaldehyde/*N*-acetyl-L-cysteine spectrophotometric assay procedure for primary amino nitrogen (K-PANOPA kit; Megazyme International Ireland Ltd.) and enzymatic assays for free ammonium ions and L-argenine, to account for the contribution of the side chain (K-LARGE kit; Megazyme International Ireland Ltd.). Final YAN calculations allowed for the fact that the primary amino group of L-argenine is assayed twice. The synthetic medium was sterilized by filtration (0.20 μm disposable sterile filter units, Nalgene, Rochester, NY) prior to use.

**Yeast.** Yeast starter cultures were prepared by adding ∼0.25 g of yeast (strain EC1118, Prise de Mousse, AB Mauri, Australia) to 25 mL of MGJM, which was incubated overnight at 28 °C with shaking. Prior to use, the starter culture was centrifuged (4000 rpm for 10 min) and then resuspended in 20 mL of sterile water. This process was repeated a further two times. The yeast culture was then adjusted to 1.0 AU at 600 nm by dilution with sterile water.

**Fermentation Conditions.** All fermentations (50 mL) were prepared under sterile conditions. Controlled fermentations were carried out by dissolving the C6 compounds in ethanol, which were then spiked into MGJM at 0, 0.125, 0.25, 0.5, 1, 2, or 4 equiv of the approximate physiological concentrations measured in the representative grape must, and then inoculated with yeast starter culture (1 mL, adjusted to 1.0 AU at 600 nm). Air locks were used to maintain an anaerobic environment. In all cases, three separate ferments for each treatment were prepared by spiking of alcohol or aldehyde solution to MGJM to afford biological triplicates. Fermentations were allowed to proceed until mass loss stabilized. Fermentation was halted by removing yeast cells by centrifugation (4000 rpm for 5 min). The clarified wines were then stored in glass at 4 °C prior to analysis.

**Headspace Volatile Analysis.** Solid-phase microextraction (SPME)−gas chromatography mass spectrometry (GCMS) was used to analyze the volatile constituents of the wines produced from the fermentation of the spiked MGJM. Aliquots of the wines (5 mL) were analyzed and diluted 1 in 2 with $H_2O$ to a final volume of 10 mL. In all cases, NaCl (3 g) was added to each SPME vial (20 mL) prior to sample addition. Samples were spiked with $D_{13}$-hexanol as an internal standard (1 in 2 dil. or neat: 9.20 μg) prior to SPME-GCMS analysis.

SPME-GCMS was carried out using an Agilent 6890 gas chromatograph equipped with a Gerstel MP2 autosampler and using an Agilent Technologies 5973N mass spectrometer for peak detection and compound identification. The autosampler was operated in SPME mode utilizing a divinylbenzene-carboxen-polydimethylsiloxane fiber (2 cm, 23-gauge, 50/30 μm DVB-CAR-PDMS, Supelco, Bellefonte, PA) for extraction. Volatile compounds were extracted using agitation (250 rpm) at 40 °C for 30 min. Chromatography was performed using a ZB-Wax column (length 30 m, 0.25 mm i.d., film thickness 0.25 μm) using helium as a carrier gas at 1.2 mL/min (constant flow). Volatiles were desorbed from the fiber in the GC inlet (220 °C) for 1 min and separated using the following temperature program: 40 °C for 1.5 min, increasing at 7 °C/min to 245 °C, and held isothermally at 245 °C for 4.5 min. The temperature of the transfer line connecting the GC and MS was held at 250 °C. Positive-ion electron impact spectra (70 eV) were recorded in scan mode (range, $m/z$ 35−350; scan rate, 4.45 scans/s).

The identity of detected volatiles was determined by comparing mass spectra with those of authentic standards and spectral libraries. A laboratory-generated library (328 compounds) as well as the U.S. National Institute of Standards and Technology-05a (NIST-05a) and the Wiley Registry seventh Edition mass spectral libraries were used for identification purposes. Compounds were considered positively identified after matching of both mass spectra and linear retention indices (LRI) with that of authentic samples. The LRI was calculated from a compounds retention time relative to the retention of a series of *n*-alkanes ($C_8$−$C_{26}$).

**Data Analysis.** The components of the samples were quantified relative to the internal standard ($D_{13}$-hexanol) using the peak area of an extracted ion. The effect of changing the concentration of the spiked component in the must on the concentration of volatiles in the headspace of the wines was analyzed by one-way analysis of variance (ANOVA) using SPSS 16.0 (SPSS Inc., Chicago, IL). When means were not significantly different across the treatments as indicated by ANOVA ($p < 0.05$), the compounds were eliminated from further analyses. When the mean peak areas of volatile compounds were found to be significantly different, Duncan's multiple range tests were performed to determine significant differences ($p < 0.05$) among the treatments.

**Table 1. Concentrations of Volatile Components Produced in Wines Made from Model Musts Spiked with Increasing Amounts of Hexan-1-ol[a]**

| | conc ($\mu$M)/grape equivalents of hexanol spiked into model must | | | | | | | spiked control |
|---|---|---|---|---|---|---|---|---|
| analyte | 0.0/0.0 | 0.25/0.125 | 0.50/0.25 | 1.00/0.50 | 2.00/1 | 4.00/2 | 8.00/4 | 2.00/1 |
| hexan-1-ol | tr | 0.19 e | 0.21 e | 0.52 d | 1.12 c | 2.20 b | 4.30 a | 1.46 |
| hexyl acetate | tr | tr | tr | tr | 0.01 c | 0.02 b | 0.03 a | tr |

[a]Values represent means ($n$ = 3), and different letters denote significant differences between treatments at $p < 0.05$. tr = trace levels of analyte were detected but were not quantifiable.

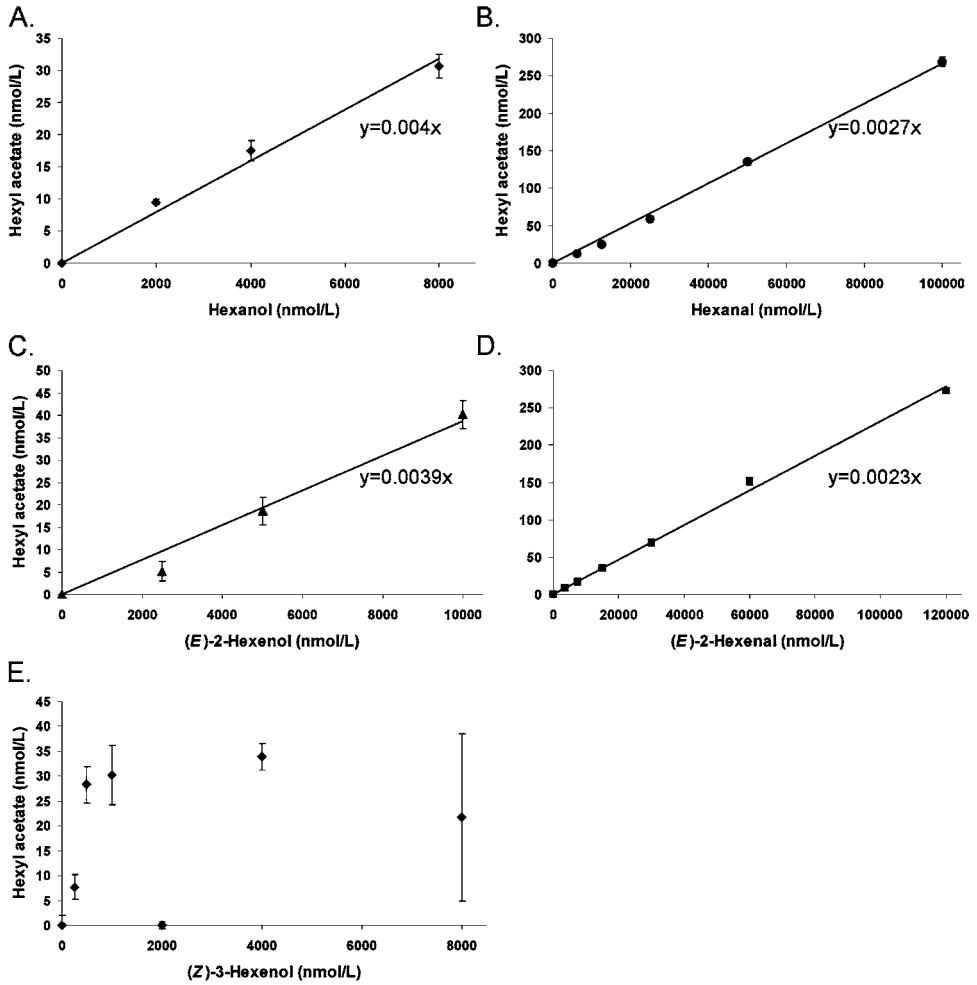


**Figure 1.** Postfermentation concentration of hexyl acetate as a function of prefermentation concentration of C6 precursor compounds. (A) Hexan-1-ol, (B) hexanal, (C) (*E*)-2-hexan-1-ol, (D) (*E*)-2-hexanal, and (E) (*Z*)-3-hexen-1-ol. Points have been plotted only in situations where hexyl acetate was quantifiable.

## RESULTS AND DISCUSSION

**Identification of Potential Precursors to Hexyl Acetate.** Previous studies[26,27,29] have shown that numerous C6 compounds are formed during the crushing of grapes, presumably through lipoxygenase activity on C18 fatty acids produced within the grape tissue.[30,31] These C6 compounds, namely, hexan-1-ol, hexanal, (*E*)-2-hexenal, (*E*)-2-hexen-1-ol, and (*Z*)-3-hexen-1-ol, have been found in grape juice musts of all varieties tested in various concentrations prior to fermentation.[26,27,29,32] This group of compounds were considered likely candidates to act as precursors to hexyl acetate through alcohol acetyl transferase (AAT) activity during yeast fermentation.[18,33]

To identify precursors to hexyl acetate, controlled model fermentations using MGJM were utilized. For this study, the model musts were spiked with potential precursors prior to inoculation and fermentation. In conducting the controlled fermentations in this fashion, the only variable being tested was the amount of precursor spiked into the must prior to inoculation. To ensure that the experiments have significance to the situation experienced during commercial winemaking, approximate physiological concentrations of the potential C6 precursors in grape juice musts were determined by SPME-GCMS analysis of a representative Cabernet Sauvignon grape juice (see refs 26 and 27 for representative concentrations of C6 compounds).

**C6 Aldehydes and Alcohols Are Precursors to Hexyl Acetate.** To determine the effect of the previously named C6 compounds on hexyl acetate production during fermentation, aliquots of MGJM were spiked with an appropriate amount of


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**Table 2. Concentrations of Volatile Components Produced in Wines Made from Model Musts Spiked with Increasing Amounts of Hexanal[a]**

| | conc ($\mu$M)/grape equivalents of hexanal spiked into model must | | | | | | | spiked control |
|---|---|---|---|---|---|---|---|---|
| analyte | 0.0/0.0 | 3.13/0.125 | 6.25/0.25 | 12.50/0.50 | 25.00/1 | 50.00/2 | 100.00/4 | 25.00/1 |
| hexan-1-ol | tr | 1.53 f | 3.34 e | 6.63 d | 13.17 c | 25.24 b | 45.42 a | tr |
| hexanal | ND | 0.02 c | 0.05 c | 0.05 c | 0.11 c | 0.20 b | 0.43 a | 7.60 |
| hexyl acetate | tr | tr | 0.01 e | 0.03 d | 0.06 c | 0.14 b | 0.27 a | tr |

[a]Values represent means ($n$ = 3), and different letters denote significant differences between treatments at $p < 0.05$. ND = not detected; tr = trace levels of analyte were detected but were not quantifiable.

**Table 3. Concentrations of Volatile Components Produced in Wines Made from Model Musts Spiked with Increasing Amounts of (*E*)-2-Hexen-1-ol[a]**

| | conc ($\mu$M)/grape equivalents of *E*-2-hexenol spiked into model must | | | | | | | spiked control |
|---|---|---|---|---|---|---|---|---|
| analyte | 0.0/0.0 | 0.31/0.125 | 0.63/0.25 | 1.25/0.50 | 2.50/1 | 5.00/2 | 10.00/4 | 2.50/1 |
| hexan-1-ol | tr | 0.13 f | 0.34 e | 0.69 d | 1.20 c | 2.42 b | 4.99 a | tr |
| hexyl acetate | tr | tr | tr | tr | 0.01 b | 0.02 b | 0.04 a | ND |
| (*E*)-2-hexen-1-ol | ND | ND | ND | ND | tr | 0.05 b | 0.16 a | 1.20 |
| (*Z*)-3-hexen-1-ol | ND | ND | ND | ND | ND | ND | tr | ND |
| (*E*)-3-hexen-1-ol | ND | ND | ND | ND | tr | tr | tr | ND |
| (*Z*)-3-hexenyl acetate | ND | ND | ND | ND | ND | ND | tr | ND |

[a]Values represent means ($n$ = 3), and different letters denote significant differences between treatments at $p < 0.05$. ND = not detected; tr = trace levels of analyte were detected but were not quantifiable.

**Table 4. Concentrations of Volatile Components Produced in Wines Made from Model Musts Spiked with Increasing Amounts of (*E*)-2-Hexenal[a]**

| | conc ($\mu$M)/grape equivalents of (*E*)-2-hexenal spiked into model must | | | | | | | spiked control |
|---|---|---|---|---|---|---|---|---|
| analyte | 0/0.00 | 3.75/0.125 | 7.5/0.25 | 15/0.50 | 30/1.0 | 60/2.00 | 120/4.00 | 30.00/1 |
| hexan-1-ol | tr | 2.25 f | 4.70 e | 9.59 d | 16.84 c | 30.71 b | 51.26 a | tr |
| hexanal | tr | tr | tr | tr | tr | tr | tr | 0.08 |
| hexyl acetate | tr | 0.01 e | 0.02 e | 0.04 d | 0.07 c | 0.15 b | 0.28 a | ND |
| (*E*)-2-hexen-1-ol | ND | ND | tr | 0.01 d | 0.11 c | 0.31 b | 0.72 a | tr |
| (*E*)-2-hexenal | ND | ND | ND | tr | tr | tr | tr | 12.21 |
| (*Z*)-3-hexen-1-ol | ND | ND | ND | ND | tr | tr | 0.03 | tr |
| ethyl (*Z*)-3-hexenoate | ND | ND | ND | ND | 0.01 c | 0.02 b | 0.03 a | ND |
| (*Z*)-3-hexenyl acetate | ND | ND | ND | ND | ND | tr | tr | tr |

[a]Values represent means ($n$ = 3), and different letters denote significant differences between treatments at $p < 0.05$. ND = not detected; tr = trace levels of analyte were detected but were not quantifiable.

alcohol or aldehyde as a solution in ethanol. The spiked musts were inoculated with yeast (*Saccharomyces cerevisiae* EC1118) and allowed to ferment to "dryness" (no further weight loss noted). Fermentation was typically finished within 12−14 days of inoculation to produce a "finished" wine. The contribution of each C6 precursor to the final volatile composition was determined using quantitative SPME-GCMS (Tables 1−5). Two control systems were used in the fermentation series. The first column of Tables 1−5 denotes yeast fermentation controls, whereby the MGJM was fermented without the addition of any C6 compound. The final column of each table represents a nonfermentative control, in which the physiological equivalent amount of the compound of interest was spiked into model wine and incubated alongside the model fermentations to estimate losses due to volatilization.

With the exception of (*Z*)-3-hexen-1-ol (Table 5), hexan-1-ol was the principle volatile C6 metabolite of the fermentations with the various C6 compounds. It was also clear from Tables 1−4 that hexan-1-ol, hexanal, (*E*)-2-hexen-1-ol, and (*E*)-2-hexenal were metabolized to hexyl acetate during yeast fermentation. Equally apparent was that postfermentation hexyl acetate concentrations in the finished wines increased proportionally to the prefermentation concentration of the C6 compound spiked into the must (Figure 1). The efficiency of the conversion from C6 precursor to hexyl acetate varied with the different precursor compounds but was very low (0.2−0.4 mol %) in all cases. Figure 1 shows graphical representations of postfermentation hexyl acetate concentrations against prefermentation concentration of precursor. The gradient of each of these functions represents the efficiency of conversion for each precursor. The different conversion efficiencies might reflect the different rates of activity of AAT with the different substrates or varying rates of diffusion of the precursors into the yeast cell. Different rates of AAT activity for different substrates have been reported in *S. cerevisiae*[34] and non- *Saccharomyces*[35] yeast strains. Seeman et al.[36] have reported that absorption of *n*-alcohols to erythrocyte membranes is dependent on chain length of the alcohol. To extend this to the current study, it would reasonable to expect that there would be differences in rates of uptake of the different precursors into the yeast cell. Alternatively, different rates of volatilisation for each C6 compound may influence the amount that enters the yeast cells during fermentation.


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**Table 5. Concentrations of Volatile Components Produced in Wines Made from Model Musts Spiked with Increasing Amounts of (*Z*)-3-Hexen-1-ol[a]**

| | conc ($\mu$M)/grape equivalents of (*Z*)-3-hexenol spiked into model must | | | | | | | spiked control |
|---|---|---|---|---|---|---|---|---|
| analyte | 0.00/0.00 | 0.25/0.125 | 0.50/0.25 | 1.00/0.50 | 2.00/1 | 4.00/2 | 8.00/4 | 2.00/1 |
| hexan-1-ol | tr | 0.08 bc | 0.12 bc | 0.21 ab | 0.04 c | 0.31 a | 0.20 ab | tr |
| hexyl acetate | tr | 0.01 ab | 0.03 ab | 0.03 a | 0.00 b | 0.03 a | 0.02 ab | tr |
| (*Z*)-3-hexen-1-ol | ND | 0.04 f | 0.17 e | 0.42 d | 1.01 c | 2.24 b | 4.61 a | 1.18 |
| (*E*)-3-hexen-1-ol | ND | tr | 0.05 e | 0.20 d | 0.53 c | 1.25 b | 2.63 a | tr |
| ethyl (*Z*)-3-hexenoate | ND | ND | ND | ND | 0.03 c | 0.07 b | 0.14 a | ND |
| (*Z*)-3-hexenyl acetate | ND | ND | ND | tr | tr | tr | tr | tr |

[a]Values represent means ($n$ = 3), and different letters denote significant differences between treatments at $p < 0.05$. ND = not detected; tr = trace levels of analyte were detected but were not quantifiable.

Hexan-1-ol would be predicted to be a direct precursor to hexyl acetate through the action of an alcohol acetyl transferase (AAT) enzyme.[18,37] However, the mechanism for the production of hexyl acetate was less clear when hexanal, (*E*)-2-hexenal, and (*E*)-2-hexen-1-ol were the substrates. Tables 2−4 indicate that a significant proportion of hexan-1-ol is formed in the fermentations spiked with these compounds. This raises questions about the order of the reduction and acetylation events that must take place during hexyl acetate formation with these substrates. Hexanal and (*E*)-2-hexenal require reduction of the aldehyde to hexan-1-ol and (*E*)-2-hexen-1-ol, respectively, before acetylation. The formation of hexyl acetate from (*E*)-2-hexen-1-ol requires both acetylation of the hydroxyl and reduction of the alkene. Whether reduction occurs first, then acetylation, or acetylation followed by reduction, could not be determined in these experiments. However, Herriaz et al.[38] observed a rapid conversion of (*E*)-2-hexen-1-ol to hexan-1-ol during the first 2 days of yeast fermentation. Accordingly, it would be reasonable to expect that reduction occurs prior to acetylation during fermentation, but the alternate sequence cannot be ruled out. Whichever sequence dominates, our results suggest that (*E*)-2-hexen-1-ol certainly acts as a grape-derived precursor to hexyl acetate.

A very different story was observed for fermentations spiked with (*Z*)-3-hexen-1-ol (Table 5). In this series of fermentations, hexyl acetate was detected above the concentrations observed in control fermentations (MGJM with no C6 compound spiked). However, the observed concentrations of hexyl acetate were not proportional to the amount of (*Z*)-3-hexen-1-ol spiked into the MGJM (Table 5). It appears that while (*Z*)-3-hexen-1-ol can act as a precursor to hexyl acetate, the mechanism by which it is used by the yeast for the production of the acetate ester is not totally dependent on the concentration of this C6 compound. This alcohol was partially recovered in the finished wines unaltered, and postfermentation concentrations of (*Z*)-3-hexenyl acetate were also measured in this series (Table 5). In ferments containing 0−0.5 grape equivalents of (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate was not detected, but this ester could be detected in trace quantities in ferments containing higher prefermentation quantities of (*Z*)-3-hexen-1-ol. This suggests that some esterification of this alcohol is possible during fermentation. However, trace quantities of (*Z*)-3-hexenyl acetate were observed in the model wine controls, so it is possible that the observation of this ester in the wines from the ferment series could be due to spontaneous esterification in solution rather than yeast-mediated esterification.

The linear increase of the postfermentation concentration of hexyl acetate (Figure 1) with respect to the precursor concentration strongly suggests that the limiting factors in hexyl acetate production are the rate of the diffusion of the precursor into the yeast cell, the rate of AAT activity, or a combination of the two as discussed earlier. As the must concentration of the precursor increases, more is available to diffuse into the yeast cell. It seems unlikely that an active transport pump is involved in the transport of the C6 precursors into the yeast cell. If this was the case, a plateau effect of the concentration of hexyl acetate relative to precursor would be expected to be observed as the rate of transfer became the rate-determining factor. This was not the case for any precursor. However, an active transport pump cannot be ruled out as it is possible that the concentrations of the precursors spiked into the must were well below that required to approach the $J_{max}$ (maximum transport rate) of any potential transport protein. Furthermore, the lack of any plateau in hexyl acetate production suggests that the substrate concentration entering the cells is not limiting the activity of the AAT enzyme or the concentrations of precursors in the yeast cell are in the linear range of activity of this enzyme.

It is worth noting that fermentations with C6 compounds spiked into them were conducted by Herraiz et al.[38] This paper principally focused upon the recovery of C6 compounds after fermentation, with little reference to hexyl acetate and other ester production, which was not discussed. However, the results presented in this previous work do support the findings of this current study. That is, that the postfermentation concentration of hexyl acetate increases as the prefermentation must concentration of the C6 precursor increases.

**C6 Compound Metabolism Is Biologically Relevant in "Real" Grape Musts.** To validate the findings of the model fermentation experiments concerning C6 compound metabolism during yeast fermentation, a similar spiking experiment was carried out using a Riesling juice in place of the MGJM. An approximate concentration of hexan-1-ol was determined in the Riesling juice. Hexan-1-ol was then spiked into musts to increase the total amount to approximately 2- and 4-fold the amount present in the juice, and these were then inoculated with yeast and allowed to ferment along with unspiked controls. As with the model must fermentation series, volatile compositions of the finished wines were determined by quantitative SPME-GCMS (Table 6).

This fermentation series mirrored those described for the model must series, which shows that the model system results are biologically relevant in real grape musts. Hexan-1-ol was the principle volatile C6 metabolite produced in these fermentations, along with smaller quantities of a variety of hexenols and acetate esters. The postfermentation concentration of hexyl acetate increased with a higher must concentration of the

**Table 6. Concentrations of Volatile Components That Are Produced in Wines Made from Riesling Grape Juice Spiked with Two Different Amounts of Hexan-1-ol[a]**

| | conc ($\mu$M)/grape equivalents of hexanol spiked into Riesling | | | spiked control |
|---|---|---|---|---|
| analyte | 0.00/1 | 5.5/2 | 16.5/4 | 5.5/1 |
| hexan-1-ol | 17.29 c | 19.57 b | 24.41 a | 5.22 |
| hexyl acetate | 0.23 c | 0.26 b | 0.40 a | 0.01 |
| (*E*)-2-hexen-1-ol | 0.07 | 0.09 | 0.09 | ND |
| (*Z*)-3-hexen-1-ol | 0.49 | 0.50 | 0.49 | ND |
| (*E*)-3-hexen-1-ol | 0.38 | 0.42 | 0.40 | ND |
| ethyl (*Z*)-3-hexenoate | 0.01 | 0.01 | 0.01 | ND |
| (*Z*)-3-hexenyl acetate | tr | tr | tr | ND |

[a]Values represent means ($n$ = 3), and different letters denote significant differences between treatments at $p < 0.05$. ND = not detected; tr = trace levels of analyte were detected but were not quantifiable.

precursor hexan-1-ol. However, unlike the model fermentation series, the increase of hexyl acetate was not directly proportional to the concentration of the spiked precursor. This is attributed to other C6 compounds already present in the must prefermentation, such that the addition of a further equivalent of hexan-1-ol to the must did not represent a doubling of the total pool of C6 precursor compounds available for esterification. Nonetheless, these results further illustrate that hexan-1-ol is indeed a precursor to hexyl acetate and that the model fermentation series results are relevant to fermentation of real grape musts.

**Physiological Concentrations of Acetic Acid, Pantothenate, and Pyruvate Concentrations Do Not Affect Hexyl Acetate Production.** The C6 compounds hexan-1-ol, (*E*)-2-hexen-1-ol, hexanal, and (*E*)-2-hexenal all contributed to the total pool of hexyl acetate produced during yeast fermentation. The assumption is that these precursors all provided the alcoholic component of hexyl acetate. Other factors involved in the esterification reaction are also important in hexyl acetate formation during fermentation. Accordingly, the effects of some potential grape-derived precursors to the acetate moiety of hexyl acetate were also studied using spiked MGJM fermentation experiments.

Acetic acid, pantothenate, and pyruvate were chosen as compounds that could influence the production of the acetate moiety of hexyl acetate. All of these compounds can act as precursors to acetyl-CoA, the activated acetate required as a substrate for AAT activity.[18] Fermentation experiments with these compounds were conducted in triplicate by spiking them into MGJM containing 2 $\mu$M hexan-1-ol at the following concentrations: acetic acid, 20, 40, 80, 160, 320, and 640 $\mu$M; pantothenate, 400, 600, 800, 1000, and 1200 $\mu$g/L; pyruvate, 1, 10, and 100 $\mu$g/L and 1, 10, and 100 mg/L. Musts were then inoculated with yeast and allowed to ferment to dryness. Postfermentation concentrations of hexyl acetate in the wines were measured using SPME-GCMS.

Under the fermentation conditions used in these experiments, none of these three compounds had any effect on hexyl acetate production at the concentrations added into the fermentations. In all cases, postfermentation concentrations of hexyl acetate were not significantly different to those of control fermentations (data not shown). There are several possible explanations for these observations. Acetic acid should enter the cells by simple diffusion under the conditions of this experiment,[39] but this compound will also be toxic to the yeast.[40] It is assumed that the acetic acid is subsequently transported out of the yeast or catabolized into a nontoxic compound but not hexyl acetate. Alternatively, the levels spiked into the medium, which are in the range found in grape musts, are insignificant in comparison to endogenous production by yeast; hence, there was no noticeable affect on acetate ester production. Likewise, pyruvate is a potential precursor to acetyl-CoA in the yeast, but it is also an intermediate in many aspects of carbon metabolism[41] and so may be shunted into several alternative pathways. Pyruvate is transported into yeast via a permease,[42] and the permease levels are down-regulated by glucose.[43] Therefore, the pyruvate spiked into the musts may only enter the yeast late in the fermentation process, by which time the levels of ethanol will be so high it would be the dominant alcohol substrate for the AAT enzyme. With regards to the addition of pantothenate, the MGJM used for these experiments contained 400 $\mu$g/L of pantothenate and an excess of YAN, both as free ammonium and amino acids. Under these conditions, pantothenate does not appear to play a rate-determining role in hexyl acetate synthesis, suggesting that acetyl-CoA production was not limiting in these experiments. The study conducted by Hagen et al.[44] determined the pantothenic acid content of a number of grape varieties from different vineyards in the Pacific Northwest of the United States. Pantothenic acid concentrations varied between 179 and 1260 $\mu$g/L. Therefore, the concentrations of pantothenate spiking in the experiments described here were physiologically significant. However, it is possible that pantothenate could play a rate-determining role in hexyl acetate formation at lower concentrations and/or in musts with low YAN.[45] The YAN concentration of the MGJM used for this study was measured at 790 ± 12 mg N/L. This concentration represents a 4−5-fold higher level of YAN than that reported by Bell and Henschke[46] required for complete fermentation (140−150 mg N/L).

These results combined with those of the C6 compound spiking experiments clearly show that the prefermentation concentration of precursor C6 alcohols and aldehydes predominately determine the postfermentation concentration of hexyl acetate in the model must system utilized in this study. Accordingly, this study suggests the total pool of hexyl acetate produced during fermentation of real grape musts will be strongly influenced by the total content of hexan-1-ol, hexanal, (*E*)-2-hexen-1-ol, and (*E*)-2-hexenal in the must prefermentation.

**Prefermentation Concentrations of Other Alcohols in Musts Determine the Postferment Concentrations of Their Acetate Esters.** As well as the previously mentioned C6 alcohols and aldehydes, grape juice musts also contain various other alcohols in varying concentrations. It was postulated that the prefermentation concentrations of these alcohols should also determine the postfermentation concentration of their corresponding acetate esters. To test this hypothesis, two further series of MGJM fermentations were prepared. Octan-1-ol and benzyl alcohol were chosen as two candidate alcohols found in grape juice musts. Increasing proportions of these alcohols were spiked into MGJM, then inoculated with yeast, and allowed to ferment to dryness (mass loss of fermentation stabilized). The postfermentation concentrations of octyl acetate and benzyl acetate were determined, along with the residual concentrations of octan-1-ol and benzyl alcohol (Tables 7 and 8). It should be noted that the concentrations selected for these spiking series were based on that of the

**Table 7. Concentrations of Volatile Components Produced in Wines Made from Model Musts Spiked with Increasing Amounts of Octan-1-ol[a]**

| | conc ($\mu$M)/equivalents of octanol spiked into model must | | | | | | | spiked control |
|---|---|---|---|---|---|---|---|---|
| analyte | 0.00/0.00 | 0.25/0.125 | 0.50/0.25 | 1.00/0.50 | 2.00/1 | 4.00/2 | 8.00/4 | 2.00/1 |
| octan-1-ol | 0.05 e | 0.08 d | 0.09 cd | 0.10 cd | 0.11 c | 0.16 b | 0.28 a | 1.72 |
| octyl acetate | ND | tr | tr | tr | tr | 0.005 b | 0.01 a | tr |

[a]Values represent means ($n$ = 3), and different letters denote significant differences between treatments at $p < 0.05$. ND = not detected; tr = trace levels of analyte were detected but were not quantifiable.

**Table 8. Concentrations of Volatile Components Produced in Wines Made from Model Musts Spiked with Increasing Amounts of Benzyl Alcohol[a]**

| | conc ($\mu$M)/equivalents of benzyl alcohol spiked into model must | | | | | | | spiked control |
|---|---|---|---|---|---|---|---|---|
| analyte | 0.00/0.00 | 0.25/0.125 | 0.50/0.25 | 1.00/0.50 | 2.00/1 | 4.00/2 | 8.00/4 | 2.00/1 |
| benzyl alcohol | ND | tr | 0.09 d | 0.16 d | 0.32 c | 0.62 b | 1.24 a | 0.81 |
| benzyl acetate | ND | tr | tr | 0.01 d | 0.02 c | 0.04 b | 0.09 a | tr |

[a]Values represent means ($n$ = 3), and different letters denote significant differences between treatments at $p < 0.05$. ND = not detected; tr = trace levels of analyte were detected but were not quantifiable.

hexan-1-ol content of a must so a direct comparison with octan-1-ol and benzyl alcohol could be established and to allow sufficient concentrations to permit accurate measurement of acetate esters formed postfermentation.

In both of these fermentation series, the postfermentation concentration of octyl acetate or benzyl acetate increased in proportion to the prefermentation concentration of the corresponding alcohol. For octan-1-ol, the conversion of the alcohol to the corresponding acetate was very low, being approximately 0.1 mol % (Table 7), but it was 1.1 mol % for benzyl acetate (Table 8). In the case of EC1118, the efficiency of conversion of the alcohols studied to their corresponding acetate esters was benzyl alcohol > hexan-1-ol > octan-1-ol. These observations support the earlier hypothesis stating that the efficiency of conversion is due to the rate of diffusion of the precursor into the yeast cytosol, the different selectivity of the enzyme for the different precursors, or a combination of the two. In a complex must, it should be remembered that there will also be competition between substrates.

Recovery of the three different alcohols postfermentation varied widely (Tables 1, 7, and 8). In the case of hexan-1-ol, recovery was around 50 mol % (based on initial concentration of the must) regardless of initial must concentrations (Table 1). Octan-1-ol recovery was much lower and decreased as the initial must concentration increased (Table 7). The rate of recovery of this alcohol decreased from approximately 20−30 mol % for 0.25−0.5 $\mu$M must concentration to 3−5 mol % for must concentrations of 2−8 $\mu$M. Postfermentation recovery of benzyl alcohol (Table 8) was very low (approximately 15 mol %) as compared to hexan-1-ol and consistent for all must concentrations. Metabolism of these alcohols to acetate esters only accounts for a small percentage of the observed losses. Undoubtedly, a significant portion of these alcohols are lost through volatilization with $CO_2$ evolution during fermentation. Volatilization can account for the loss of hexan-1-ol and benzyl alcohol, as control experiments in model wine showed that hexan-1-ol and benzyl alcohol were recovered in these controls in 75 and 40 mol %, respectively. Volatilization does not account for the greater proportion of octan-1-ol lost during fermentation as compared to the other alcohols. Octan-1-ol is larger and less volatile than hexan-1-ol. Consequently, the loss of octan-1-ol through volatilization should be lower than that observed for hexan-1-ol, which is reflected in the control experiments. Recovery of octanol in controls was 85 mol %. Other pathways for metabolism of octan-1-ol to other unknown, potentially nonvolatile, metabolites appear to be operating in these circumstances. The results also suggest that these pathways are highly sensitive to the initial must concentration of the alcohol. Alternatively, the octan-1-ol may be sequestered in the cell walls or membranes of the yeast and are thereby lost when the yeast was removed from the wine by centrifugation.

There are some intriguing sensory implications of these results. The acetate esters discussed in this study are often described as having fruity, red berry aromas,[17,28] which are generally considered positive contributors to wine flavor. Conversely, the precursor alcohols are described as green/herbaceous (hexanol), to green/fatty (octanol),[1,47] generally negative characteristics (although their presence can add complexity to a wine). However, these results imply that to get the desirable aroma compounds (the esters), the less desirable precursor alcohols or aldehydes must also be present. The overall effect on wine flavor of these compounds will depend strongly on the relative ratio of the precursors to esters in the final wine and how that relative ratio affects interactions with other volatile and nonvolatile compounds in the wine matrix. Pineau et al.[17] showed that esters can display synergistic effects whereby the overall fruit aroma of a wine can be influenced by the total ester content. Additionally, the same study demonstrated that small changes in ester concentration can alter the overall aroma of a wine, and individual esters can exert aroma effects even when present below threshold values. With this in mind, differences in precursor concentrations prior to fermentation can effect changes in concentration of their corresponding acetate esters, which could result in differences in overall aroma of wines.

It is important to note that in all of the precursor spiking ferments, alcohol/aldehyde to acetate ester conversion was very low. Using EC1118 as the fermentation yeast, conversion was only approximately 0.2−1 mol % depending on the precursor. The use of other yeast strains will likely alter these conversion ratios.[48] However, it is still likely that the must concentration of the precursor compounds will determine the postfermentation concentration of the corresponding acetate esters regardless of the yeast strain used for fermentation. Use of different yeast strains will likely have an effect on wine flavor and aroma. If

different ratios of precursor to acetate ester are achieved postfermentation using different yeast strains, the finished wines are liable to have different aroma characteristics.

**Implications of This Study.** The experiments described in this paper highlight the grape or must dependence of the production of acetate esters of hexanol, octanol, and benzyl alcohol during yeast fermentation in model must media. The simple fermentation experiments used further exemplify the utility of MGJM spiking studies to determine factors involved in volatile compound production during yeast fermentation. A clear, linear relationship exists between the prefermentation must concentrations of the alcohol/aldehyde substrates and the postfermentation concentration of the corresponding acetate esters in these model ferment series. As the prefermentation concentration of the alcohol/aldehyde increases, there is a proportional increase in the postfermentation concentration of the acetate ester. In the case of hexyl acetate, multiple C6 alcohols and aldehydes are grape-derived precursors to this ester, so the postferment concentration of this ester will be influenced upon the sum of the precursors present in the initial musts of real grape fermentations. It should be noted, however, that there could be other, unidentified precursors present in the must that could be metabolized to hexyl acetate during fermentation of real grape musts.

Under the conditions used in this study, the initial precursor concentration is the limiting factor in the final concentration of the acetate ester in question. To extrapolate these results to a real grape must, this suggests that the grape plays a major role in formation of the three yeast-derived acetate esters examined in this research: hexyl acetate, octyl acetate, and benzyl acetate. Armed with this knowledge, winemakers could use prefermentation concentrations of the precursors discussed in this study as a guide for acetate ester content after fermentation. With some idea of ester potential in a grape juice must, the winemaker could alter winemaking variables such as yeast strain and/or fermentation temperatures to produce a wine with an appropriate level of fruitiness for the desired wine style. Control of wine style could also potentially start in the vineyard through understanding the viticultural practices affecting the formation of alcohol and aldehyde precursors in grape juice musts. As such, viticultural practices could be adjusted to produce grape juice musts that contain either high or low concentrations of acetate ester precursors to fit the desired wine style.

While grape musts and wine have been the principle focus of this study, the use of model must ferments means these results can be applied to other fermented beverages such as beer or cider. If the respective prefermentation musts of these beverages contain any alcohol or aldehyde precursors, then these will influence the postfermentation concentrations of the associated acetate esters. How this effects the sensory attributes of these beverages will depend strongly upon other aroma active compounds present in the beverage and how these compounds interact with the acetate esters.

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## ABBREVIATIONS USED

GCMS, gas chromatography mass spectrometry; LRI, linear retention index; MGJM, model grape juice medium; SPME, solid-phase microextraction; YAN, yeast assimilable nitrogen; YNB, yeast nitrogen base; AAT, alcohol acetyl transferase

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