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# Shiraz Wines Made from Grape Berries (*Vitis vinifera*) Delayed in Ripening by Plant Growth Regulator Treatment Have Elevated Rotundone Concentrations and "Pepper" Flavor and Aroma

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Supporting Information

**ABSTRACT:** Preveraison treatment of Shiraz berries with either 1-naphthaleneacetic acid (NAA) or Ethrel delayed the onset of ripening and harvest. NAA was more effective than Ethrel, delaying harvest by 23 days, compared to 6 days for Ethrel. Sensory analysis of wines from NAA-treated fruit showed significant differences in 10 attributes, including higher "pepper" flavor and aroma compared to those of the control wines. A nontargeted analysis of headspace volatiles revealed modest differences between wines made from control and NAA- or Ethrel-treated berries. However, the concentration of rotundone, the metabolite responsible for the pepper character, was below the level of detection by solid phase microextraction−gas chromatography−mass spectrometry in control wines, low in Ethrel wines (2 ng/L), and much higher in NAA wines (29 ng/L). Thus, NAA, and to a lesser extent Ethrel, treatment of grapes during the preveraison period can delay ripening and enhance rotundone concentrations in Shiraz fruit, thereby enhancing wine "peppery" attributes.

**KEYWORDS:** *berry ripening, 1-naphthaleneacetic acid, Ethrel, wine, aroma, flavor, rotundone*

## INTRODUCTION

Endogenous hormones play important roles in the control of grape berry development and, in particular, ripening.[1] The application of plant growth regulators (PGRs) can be used to manipulate the timing of veraison and thereby the harvest date.[1−6] Some PGRs, such as abscisic acid, can promote ripening, while others, such as auxins, can delay ripening; therefore, PGRs are useful tools with which to manipulate berry development.[1−6] There is a growing body of evidence that the rise in air temperatures associated with climate change has advanced grape berry ripening, resulting in early harvests and a shortening of the harvest season.[7] One useful outcome of an improved ability to manipulate ripening would be to alleviate winery intake scheduling issues arising from compressed harvest seasons. For example, delaying the ripening or harvest of part of a winery's intake would spread the workload associated with processing over a longer period. The application of PGRs may also affect berry composition and therefore wine flavor and aroma, either directly or as a consequence of the altered timing of ripening and harvest.[8]

In previous studies, the synthetic auxin 1-naphthaleneacetic acid (NAA) was used to delay the ripening of Shiraz and Riesling grapes.[8,9] NAA was used in preference to the grape berry endogenous auxin, indole-3-acetic acid (IAA), as IAA was shown to be rapidly inactivated and ineffective in delaying ripening.[2] It was shown that NAA caused a significant delay in the commencement of ripening and harvest date. In the experiment with Shiraz, wines made from NAA-treated berries had small differences in the concentration of flavor/aroma volatiles as measured by SPME−GC−MS analysis and could not be distinguished from the control wines by sensory analysis.[8] In the experiment with Riesling, only modest differences in flavor/aroma volatiles were measured, but as opposed to the Shiraz example, a sensory panel could distinguish the wines made from NAA-treated fruit from those made from control fruit.[9] Ethrel application, which gives rise to ethylene, during the preripening period has also been shown to delay ripening.[3,4,6] This is most likely due to an induction of IAA accumulation that occurs upon Ethrel application.[5] In contrast, ripening can be advanced by the preveraison application of aminoethoxyvinylglycine, an inhibitor of both ethylene and auxin biosynthesis.[6]

The grape variety Shiraz is grown around the world and produces red wines having a range of styles depending on climate, viticultural practice, and winemaking practice. In a number of Shiraz wines, a "pepper" character (referring to the spice and not the fruit otherwise known as capsicum) is seen as a distinctive attribute.[10] A compound found in grapes and wine that confers "pepper" taste and aroma, first discovered in Shiraz, has been identified as a bicyclic sesquiterpene, called rotundone.[11] The detection threshold of rotundone in red wine has been reported to be 16 ng/L, and approximately 80% of participants of a sensory panel were able to detect this character. In addition to Shiraz, rotundone has been detected at varying concentrations in a range of red varieties, for example,

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Cabernet Sauvignon, Durif, Mourvedre,[11] Schioppettino, and Vespolina, and in a white variety, Grüner Veltliner.[12] Schioppettino, Vespolina, and Grüner Veltliner are noted for their very "peppery" character and have correspondingly high concentrations of rotundone.[12] The concentration of rotundone in berries is extremely low at veraison but increases rapidly during ripening to reach maximal levels at harvest.[13] Studies of a range of varieties, including Shiraz, have shown that rotundone concentrations vary considerably between seasons and vineyard locations and even within vineyards, but as yet, there are no definitive data regarding the causal factors for this variation.[11−14]

In this paper, we describe the use of two different PGRs (Ethrel and NAA) to delay Shiraz berry ripening and how these treatments altered the profiles of a range of volatile flavor/aroma metabolites, the concentrations of rotundone and the sensorial properties of wine made from treated and control fruit.

## MATERIALS AND METHODS

**NAA and Ethrel Treatment of Field-Grown Shiraz Berries.** *Vitis vinifera* L. cv Shiraz vines, on own roots, were grown on well-drained soil, with vertical shoot positioning trellising, at Hahndorf in the Adelaide Hills (−35°.02, 138°.84, elevation 400 m). Natural rainfall was supplemented by limited drip irrigation as required. Bunches were sprayed to runoff during the preveraison period with 50 mg/L NAA (Gibco BRL Life Technologies, Grand Island, NY) in 0.1% (v/v) Chemwet 1000 (Nufarm, Laverton North, VIC, Australia), (2-chloroethyl)phosphonic acid as Ethrel at 300 μL/L (Bayer Crop Science, East Hawthorn, VIC, Australia) in 0.1% (v/v) Chemwet 1000, or a 0.1% (v/v) Chemwet 1000 solution alone (control). Spray dates were as follows: for control and Ethrel, January 16, 2012 (8 days preveraison of control berries), and January 23, 2012 (1 day preveraison of control berries); for NAA treatment, January 23, 2012, and January 27, 2012 (3 days postveraison of control berries). Veraison was defined as the sample date previous to the first sample showing an increase in Brix value of more than two degrees per week. There was no precipitation during the 48 h period after each spray treatment. The trial was of a randomized triplicate design, and the sample size per replicate was 400 bunches (1200 bunches per treatment). Samples of 60 randomly harvested berries per replicate were taken throughout development (the sampling dates are indicated in Figure 2). Sampling was completed between 09:30 and 14:30 h. Berries were weighed, and total soluble solids (TSS) were measured for each of these replicates as described below. Anthocyanins were measured at two time points, February 14, 2012, and at harvest (which was different for each of the treatments), as described below. Minimum and maximum air temperatures (Figure S1 of the Supporting Information) for a site located near the vineyard (−35°.07, 138°.84) were obtained from the Australian Bureau of Meteorology (www.bom.gov.au).

**Determination of Anthocyanin and Total Soluble Solid Levels.** Frozen whole berries were ground to a powder using an IKA A11 basic analytical mill (IKA, Staufen, Germany). For the measurement of TSS (degrees Brix), 100 mg of berry powder was thawed on ice, the tissue was pelleted by centrifugation at 18000*g* for 5 min, and the supernatant was analyzed with an RFM710 digital refractometer (Bellingham Stanley, Tunbridge Wells, U.K.). For anthocyanin determination, 300 mg of a powdered sample was added to 1.5 mL of MeOH containing 1% (v/v) HCl. Anthocyanins were extracted at room temperature in the dark on a rotating mixer for 1 h. The tissue was pelleted by centrifugation at 18000*g* for 15 min and the supernatant retained. Depending on the developmental stage, the supernatant was diluted up to 20-fold with MeOH and 1% (v/v) HCl. Total anthocyanins were measured spectrophotometrically by reading the absorbance at 520 nm immediately following centrifugation. One-way ANOVA followed by Duncan's post hoc test was performed using IBM SPSS version 20 (IBM, Armonk, NY).

**Small Scale Winemaking.** Small scale winemaking was conducted by WIC Winemaking Services (Urrbrae, SA, Australia) using the following protocol. Control, Ethrel-treated, and NAA-treated fruit (20 kg for each of three replicates per treatment) were harvested at the times indicated in Figure 2. Harvested fruit was placed at 0 °C for 12 h, and the $SO_2$ concentrations were adjusted to 50 ppm during crushing and destemming. Yeast strain EC1118 (Lallemand, Edwardstown, SA, Australia) was added to a concentration of 200 ppm. The ferment was conducted on skins at 18−20 °C, and diammonium phosphate was added as required to a maximal concentration of 400 ppm. The cap was plunged 20 times twice daily. When the must reached 2°Baume, the must was pressed and then fermented to dryness, following which it was racked. $SO_2$ was added to a concentration of 60 ppm, and the wine was cold stabilized at 0 °C for 21 days. The wine was again racked, and $SO_2$ concentrations were adjusted to 80 ppm before being filtered and bottled with 30 × 60 Stelvin closures (Amcor, Hawthorn, VIC, Australia).

**Sensory Analysis of Small Scale Wines.** Detailed sensory profiles of all wines were generated by descriptive analysis (DA) conducted at the University of Adelaide sensory laboratory, which complies with international standards for the design of test rooms (ISO 8589:1988). The trained DA panel consisted of 12 members (eight female and four male, mean age of 36 years, ranging from 24 to 57) and underwent three 2 h training sessions, including evaluation of all wine samples before final assessment. The panel generated a standard list of vocabulary terms to profile the differences between the wines for appearance, aroma, palate, mouthfeel, and aftertaste. Reference standards (Table S2 of the Supporting Information) were developed to help clarify some of the aroma sensory attributes and ensure full agreement across assessors. The DA final assessment was conducted in triplicate in individual booths with panel members tasting up to 14 samples per day. Panelists received a sample volume of 30 mL served at 21 °C in 214 mL standardized tasting wine glasses (ISO 3591:1977). Each wine glass was covered with a watch glass to prevent headspace loss, and samples were poured immediately before being served to the assessor. Samples were blind-coded with random three-digit codes, and the order of sample assessment was randomized to account for first-order and carryover effects. Processed water crackers and water were consumed between samples to minimize carryover effects, and an interstimulus interval of at least 1 min was chosen as a suitable time between samples with a 5 min interval after flights of six wines. Panelists had access to and were encouraged to use all reference samples throughout final assessment. The experimental design was produced using the design generation package Design Express (Qi Statistics, Reading, U.K.). Attributes were rated on 0−15 line scales, with indented extreme end word anchors for each descriptive term. Data were recorded and stored using Fizz sensory data acquisition software (Biosystèmes, Couternon, France).

ANOVA was conducted to test the effects of Judge, Sample, Replicate, and all two-way interactions for each sensory attribute using a pseudomixed model with the Judge × Sample interaction as a denominator. IBM SPSS version 20 (IBM) was used for these analyses.

**Nontargeted Headspace Volatile Analysis.** SPME−GC−MS was used to analyze the volatile constituents of the wines produced from the control and treated fruit. Aliquots of the wines were analyzed at two different concentrations, 1:100 or 1:2 diluted with $H_2O$ to a final volume of 10 mL. Three grams of NaCl was added to each SPME vial (20 mL) prior to sample addition.

The extraction and chromatographic conditions were identical to those described by Boss et al.[15] 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-11 (NIST-11) and the Wiley Registry 9th 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 those of authentic samples. LRI was calculated from a compound's retention time relative to the retention of a series of *n*-alkanes ($C_8$−$C_{26}$). Other compounds


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were tentatively identified on the basis of comparison with mass spectral libraries and published LRI, or comparisons with mass spectral libraries alone.

The components of the samples were quantified using Chemstation (Agilent, Forest Hill, VIC, Australia) relative to the relevant internal standard ($d_{13}$-hexanol, $d_{11}$-hexanoic acid, $d_{16}$-octanal, methyl nonanoate, or $d_3$-linalool) using the peak area of an extracted ion.

The effect of applying Ethrel and NAA to bunches on the concentration of volatiles in the headspace of the wines was analyzed by ANOVA using SPSS version 20 (IBM).

**Synthesis of Rotundone and $d_5$-Rotundone.** Guaiacwood essential oil was purchased from Auroma (Hallam, VIC, Australia). All reagents were purchased from commercial sources and used directly if no specific purification is mentioned.

The synthesis of rotundone (**4**) and $d_5$-rotundone (**5**) began with the synthesis of $\alpha$-guaiene (**3**), which was prepared from guaiol (**1**) via acetylation and pyrolysis as described by Huang et al.[16] Rotundone (**4**) was then synthesized by one-step allylic oxidation of $\alpha$-guaiene (**3**) by pyridinium dichromate (PDC) and *tert*-butyl hydroperoxide (TBHP) as described by Huang et al. (Figure 1).[17] $d_5$-Rotundone was synthesized by deuterium exchanged of rotundone with $d_6$-ethanol as described by Huang et al.[18]

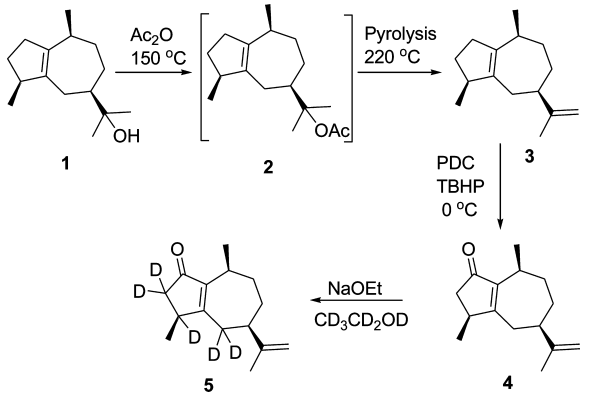


**Figure 1.** Synthesis of rotundone and $d_5$-rotundone from guaiol.

**Rotundone Quantification.** The method used for the extraction and analysis of rotundone was derived from ref 19. Styrene-divinylbenzene SPE cartridges (SDB-L, 500 mg/6 mL; Phenomenex, Lane Cove, NSW, Australia) were conditioned with a 10 mL *n*-pentane/ethyl acetate solvent (4:1), followed by 6 mL of methanol and then 6 mL of model wine (12% ethanol and 2 g/L potassium hydrogen tartrate, buffered to pH 3.2 with tartaric acid). A 100 mL aliquot of wine, containing 24 ng of $d_5$-rotundone in 100 $\mu$L of ethanol as an internal standard, was loaded onto the SPE cartridge. The cartridge was then washed with 10 mL of water followed by 2 mL of *n*-pentane and finally eluted with 10 mL of *n*-pentane/ethyl acetate solvent (9:1). This eluent was dried under a stream of $N_2$ and then redissolved in 1 mL of ethanol. The extract was added to a 20 mL amber SPME vial [Chromacol, Biolab (Aust) Ltd., Clayton, VIC, Australia] with 13 mL of aqueous tartrate buffer (2 g/L potassium hydrogen tartrate, buffered to pH 3.2 with tartaric acid) and analyzed by SPME−GC−MS.

SPME−GC−MS analysis was performed using an Agilent Technologies 7890A gas chromatograph coupled to a 5975C mass spectrometer with a MPS2 autosampler (Gerstel, Mülheim an der Ruhr, Germany). A polydimethylsiloxane/divinylbenzene 65 $\mu$m fiber (Supelco, Bellefonte, PA) was immersed in the sample for 60 min at 40 °C with agitation and then was desorbed in the inlet at 240 °C for 1 min in pulsed-splitless mode. A pressure pulse of 25.0 psi was applied for 30 s, and the flow was split with a total flow of 50 mL/min after 1 min. The fiber was cleaned for 4 min prior to extraction and also after desorption in a Gerstel fiber bake-out station at a temperature of 240 °C.

GC separation was performed on a 30 m ZB-Wax capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 $\mu$m (Phenomenex). Ultra High Purity Helium was used as a carrier gas with a constant flow rate of 1.0 mL/min. The oven temperature was initially held at 80 °C for 1 min and then increased to 220 °C at a rate of 3 °C/min, before being increased to 245 °C at a rate of 40 °C/min and then held at this final temperature for 10 min. The mass spectrometer transfer line was held at 250 °C. Selected ion monitoring (SIM) mode was used with ions at $m/z$ 223 and 218 as the selected ions for quantification of $d_5$-rotundone and rotundone, respectively, and ions at $m/z$ 147, 161, and 203 as the qualifying ions for rotundone and ions at $m/z$ 147, 161, and 208 as the qualifying ions for $d_5$-rotundone (dwell time of 30 ms, electron impact of 70 eV). The analyses were performed in triplicate for each wine.

A Shiraz bag in box wine was selected for the preparation of the calibration curves as it was found to contain no rotundone by the preceding method. The wine was spiked in triplicate to give rotundone concentrations of 0, 1.2, 6, 12, 30, and 60 ng/L, and all samples were then analyzed as outlined above. The calibration was linear throughout the range with a correlation coefficient of 0.9996 and relative standard deviations of <5%. Using this method, the limit of detection for rotundone was 0.8 ng/L and the limit of quantification was 2.7 ng/L.

## RESULTS

**Both Ethrel and NAA Treatments Delay Shiraz Berry Ripening.** The preveraison treatment of Shiraz berries with either Ethrel or NAA delayed the initiation of the phase of rapid increase in TSS and, by inference, sugar accumulation (Figure 2). After these differences in the onset of ripening, the rate of

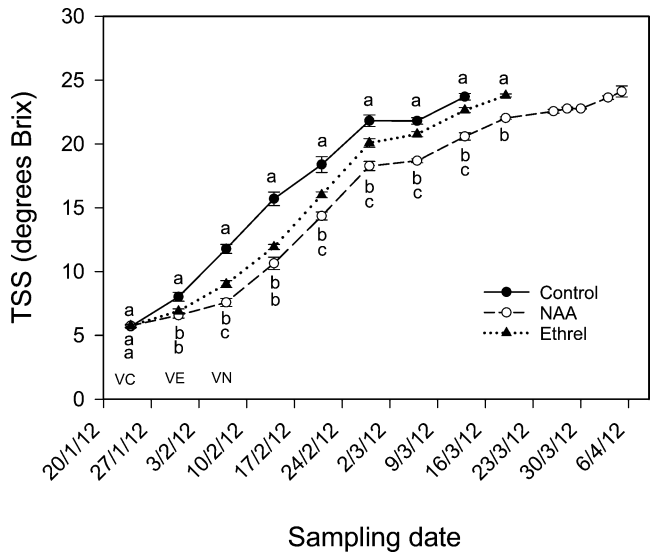


**Figure 2.** Effect of Ethrel and NAA treatments on total soluble solid (TSS) levels, measured as degrees Brix. All data represent means ± STERR ($n$ = 3), and different letters denote significant differences between treatments at $p < 0.05$ using one-way ANOVA followed by Duncan's post hoc test. VC, VE, and VN indicate the time of veraison for control, Ethrel-treated, and NAA-treated fruit, respectively.

TSS increase was similar for all fruit, as can be seen from the similar slopes of the curves in Figure 2. At most of the time points after the first sampling, the TSS levels of all three treatments were significantly different ($p < 0.05$). The delay resulting from Ethrel treatment was less pronounced than that following NAA treatment. The fruit from all three treatments were harvested for winemaking at approximately the same mean TSS value [24.1°Brix for control, 24.0°Brix for Ethrel treatment, and 24.1°Brix for NAA treatment (ANOVA $p$ =


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0.961)]. The harvest of Ethrel- and NAA-treated fruit was delayed by 6 and 23 days, respectively. Despite the changes in ripening resulting from the treatments, the final mean berry weights for all three at harvest were similar [1.33 g/berry for control, 1.41 g/berry for Ethrel treatment, and 1.39 g/berry for NAA treatment (ANOVA $p = 0.583$)]. Anthocyanin accumulation was delayed by Ethrel and NAA treatments. Three weeks after veraison of the control fruit (February 14, 2012), control berries contained a significantly larger amount of anthocyanins than Ethrel- and NAA-treated fruit, which reflects the differences in soluble solid content. In contrast, the anthocyanin concentrations in berries of similar TSS levels (approximately 24°Brix) at harvest were not significantly different (Figure 3). There was also no significant difference

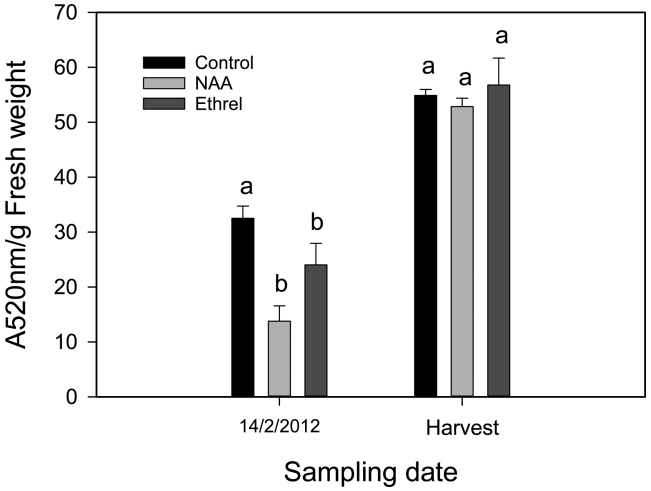


**Figure 3.** Effect of Ethrel and NAA treatments on anthocyanin accumulation ($A_{520}$). All data represent means ± STERR ($n = 3$), and different letters denote significant differences between treatments at $p < 0.05$ using one-way ANOVA followed by Duncan's post hoc test.

in titratable acidity at harvest [6.4 g/L for control, 6.7 g/L for NAA treatment, and 6.4 g/L for Ethrel treatment (ANOVA $p = 0.448$)]. From this, it appears that changes in both primary and secondary metabolism that occur during ripening were delayed by the preveraison application of Ethrel and, to a larger extent, NAA.

Wines were made from control, Ethrel-treated, and NAA-treated fruit for sensory and biochemical analysis. At the completion of winemaking, various basic measurements of the wine properties were taken. Small but statistically significant differences were observed in percent alcohol, acetic acid, pH, and titratable acidity (Table 1); these are unlikely to have an impact on the sensory properties of the wine.

**Table 1. Measurements of Percent Alcohol, Acetic Acid, pH and T.A. of Wines at Bottling[a]**

| treatment | % alcohol (v/v) | acetic acid (g/L) | pH | T.A. (g/L of $H^2T$) |
|---|---|---|---|---|
| control | 14.6 a | 0.36 a | 3.72 b | 6.8 a |
| NAA | 14.2 c | 0.27 b | 3.85 a | 6.6 ab |
| Ethrel | 14.4 b | 0.30 b | 3.80 a | 6.3 b |

[a]Values represent means ($n = 3$), and different letters denote significant differences between treatments at $p < 0.05$ using one-way ANOVA followed by Duncan's post hoc test.

**Descriptive Sensory Analysis of the Wine.** Twenty-four wine sensory attributes were scored across the wines using a trained panel. Two were attributes related to appearance, and eight were related to odor. Eleven were palate characteristics, and three measured wine mouthfeel properties. Of these, significant differences were identified between the wines for the two appearance attributes, four aroma attributes, three palate attributes, and one mouthfeel attribute (Table 2). Those

**Table 2. Sensory Attributes Found To Be Significantly Different amongs the Wines through Descriptive Analysis[a]**

| attribute | control | ethrel-treated | NAA-treated | $p$ value |
|---|---|---|---|---|
| color | 7.59 b | 6.03 c | 9.06 a | 0.001 |
| transparency | 8.50 b | 5.61 c | 9.70 a | <0.001 |
| dark fruit aroma | 8.05 b | 6.91 c | 9.40 a | 0.003 |
| red confection aroma | 6.53 a | 6.54 a | 4.95 b | 0.033 |
| earthy/dusty aroma | 1.84 b | 2.63 ab | 3.46 a | 0.007 |
| pepper aroma | 2.99 b | 2.63 b | 6.01 a | 0.003 |
| red berry flavor | 4.66 b | 5.71 a | 3.99 b | 0.025 |
| earthy/dusty flavor | 2.18 b | 2.97 ab | 3.65 a | 0.044 |
| pepper flavor | 4.41 b | 4.58 b | 7.59 a | <0.001 |
| tannin quantity | 8.89 a | 8.00 ab | 7.39 b | 0.015 |

[a]Values represent means ($n = 3$), and different letters denote significant differences between treatments at $p < 0.05$ using one-way ANOVA followed by Duncan's post hoc test. The $p$ value represents the "sample" effect (i.e., treatment) on the attribute.

attributes that were not significantly different because of the treatment of the fruit are listed in Table S3 of the Supporting Information. Wines produced from grapes treated with NAA were found to have more "color" and less "transparency" than those from the control and Ethrel-treated fruit (Table 2). Other sensory attributes more associated with the wines from the NAA-treated fruit included "dark fruit" and "pepper" aromas and "pepper" flavor on the palate (Table 2). It was also observed that scores for "red confection" odor and "red berry" flavor were significantly lower in the wine produced from NAA-treated grapes than in the wine from control and Ethrel-treated samples (Table 2). "Earthy/dusty" aroma and flavor were scored higher in the wines produced from the NAA treatment than in the control wines but were not significantly different from those of the wines from the Ethrel treatment (Table 2). In contrast, "tannin quantity" was found to be significantly lower in the wines from the NAA-treated fruit than in control wines but was not different from that of the wine from the Ethrel-treated bunches (Table 2). The Ethrel treatment also had a significant effect on wine attributes with significantly higher scores than the control and NAA wines for "red berry" flavor, but the lowest scores for wine "color", "transparency", and "dark fruit" aroma.

**Analysis of Wine Volatiles.** Significant differences were observed in the volatile profiles of the wines as determined using headspace SPME−GC−MS analysis. Of the 164 compounds quantified, eight were found in larger amounts in the headspace of the wines made from NAA-treated grapes compared to the control wines and 17 were more abundant in the control wines than in those made from the NAA-treated fruit (Table 3). No compounds were found in significantly greater concentrations in the wine from the Ethrel treatment compared with the other two treatments, but levels of two compounds (ethyl salicylate and hotrienol) were found to be lower in these wines than in wines from both the control and


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**Table 3. Volatile Compounds Found To Be Significantly Different in the Headspace of the Wines Produced from NAA-Treated, Ethrel-Treated, and Control Berries**

| compound | LRI[a] | compound ID[b] | control[c] | Ethrel | NAA |
|---|---|---|---|---|---|
| More Abundant in the Headspace of NAA-Treated Wines vs Control Wines | | | | | |
| α-calacorene | 1901 | B | 0.039 b | 0.062 b | 0.184 a |
| cadalene | 2215 | B | 0.014 b | 0.014 b | 0.033 a |
| 3-ethoxy-1-propanol | 1356 | A | 0.032 b | 0.039 ab | 0.051 a |
| ethenyl benzene | 1241 | A | 3.445 b | 3.906 b | 4.912 a |
| methionol | 1696 | A | 0.106 b | 0.123 ab | 0.135 a |
| ethyl salicylate | 1794 | B | 0.004 b | 0.002 c | 0.005 a |
| phenylethyl butyrate | 1946 | A | 0.013 b | 0.013 b | 0.016 a |
| 1,1,6-trimethyl-1,2-dihydronaphthalene | 1727 | B | 0.058 b | 0.061 b | 0.069 a |
| More Abundant in the Headspace of Control Wines vs NAA-Treated and/or Ethrel-Treated Wines | | | | | |
| (Z)-3-hexenyl acetate | 1294 | A | 0.035 a | 0.015 b | 0.016 b |
| isoamyl isovalerate | 1284 | B | 0.015 a | 0.013 ab | 0.007 b |
| hexyl acetate | 1260 | A | 1.862 a | 1.037 b | 0.936 b |
| 2-heptyl acetate | 1252 | B | 0.016 a | 0.006 b | 0.009 ab |
| (Z)-3-hexen-1-ol | 1365 | A | 0.268 a | 0.197 b | 0.154 c |
| ethyl (E)-2-hexenoate | 1330 | A | 0.080 a | 0.053 b | 0.052 b |
| theaspirane A | 1522 | B | 0.028 a | 0.019 b | 0.019 b |
| ethyl (Z)-3-hexenoate | 1287 | A | 0.102 a | 0.061 b | 0.071 b |
| (E)-3-hexen-1-ol | 1345 | A | 0.021 a | 0.015 b | 0.015 b |
| diethyl malate | 2043 | A | 0.035 a | 0.021 b | 0.025 b |
| 2-heptanol | 1308 | A | 0.029 a | 0.025 b | 0.021 c |
| 1-hexanol | 1335 | A | 2.104 a | 1.809 b | 1.591 c |
| 2-ethyl furoate | 1605 | A | 0.049 a | 0.044 a | 0.037 b |
| 1-penten-3-ol | 1157 | A | 0.021 a | 0.016 b | 0.017 b |
| 4-methyl-1-pentanol | 1296 | A | 0.119 a | 0.104 b | 0.096 b |
| theaspirane B | 1485 | B | 0.026 a | 0.019 b | 0.021 b |
| citronellyl acetate | 1646 | A | 0.006 a | 0.005 b | 0.005 b |
| α-terpineol | 1677 | A | 0.465 a | 0.406 b | 0.386 b |
| 2-phenylethanol[d] | 1910 | A | 0.305 a | 0.253 b | 0.264 ab |
| hotrienol | 1591 | B | 0.006 a | 0.005 b | 0.006 a |

[a]LRI (linear retention indices) calculated from retention of the compound relative to the retention of a series of *n*-alkanes ($C_8$–$C_{26}$). [b]Abbreviations: A, identity confirmed by matching mass spectra and LRI with that of authentic standards; B, tentative assignment based upon comparison with mass spectral libraries and published LRIs. [c]Values represent means ($n = 3$) of the areas under the peaks of a selected ion relative to the relevant internal standard. Different letters denote significant differences between treatments at $p < 0.05$ using one-way ANOVA followed by Duncan's post hoc test. [d]Compound quantified in the 1:100 dilution.

NAA-treated fruit (Table 3). However, the abundances of 18 compounds were lower in the wines produced from Ethrel-treated grapes than in those produced from control grapes (Table 3). The concentrations of 2-heptanol, 1-hexanol, and (Z)-3-hexen-1-ol were intermediate in the Ethrel treatment wines, being lowest after NAA treatment (Table 3).

Several compounds that are derived from the lipoxygenase pathway were present in lower abundances in the wines produced from NAA-treated grapes than in the control wines. These were 1-hexanol, (E)-3-hexen-1-ol, (Z)-3-hexen-1-ol, and the esters hexyl acetate, ethyl (Z)-3-hexenoate, ethyl (E)-2-hexenoate, and (Z)-3-hexenyl acetate, which have been shown to derive from $C_6$ alcohols and aldehydes produced by the lipoxygenase pathway.[20] Other lipid oxidation products with levels higher in control wines than in those produced from NAA-treated berries were 2-heptanol and 1-penten-3-ol. In general, the compounds that were significantly more abundant in the headspace of the control wines compared with those from the NAA treatment were less than 2-fold different, the exceptions being isoamyl isovalerate and (Z)-3-hexenyl acetate, with levels that were slightly more than 2-fold higher in the control wines than the NAA treatment wines.

Some aromatic compounds were more abundant in the wines from the NAA treatment than in the control wines. These were ethenyl benzene, ethyl salicylate, phenylethyl butyrate, and 1,1,6-trimethyl-1,2-dihydronaphthalene, the latter being derived from the degradation of carotenoids and the remainder from benzenoid metabolism. None of these compounds were more than 2-fold different in abundance between the wines.

**The Concentration of Some Sesquiterpenes Is Elevated by NAA Treatment.** Among the compounds found to be present at higher concentrations in the headspace of the wine from NAA-treated fruit were two sesquiterpenes tentatively identified as α-calacorene and cadalene. These volatile compounds were found in concentrations 4.7- and 2.4-fold higher, respectively, in the wines produced from the NAA-treated berries than in the control wines (Table 3). The compound responsible for pepper character in Shiraz wines has been identified as the sesquiterpene rotundone, which is sensorially active at very low concentrations.[11] Given that these two compounds had mass spectra typical of sesquiterpenes and that terpene synthase enzymes can make multiple products,[21] we decided to analyze the concentration of rotundone in the wines using a targeted analytical method. Rotundone could not be detected in the control wines, was present at low


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concentrations (2 ± 0.4 ng/L) in the wine made from Ethrel-treated fruit, but was found at 14.5-fold higher concentrations (29 ± 1.6 ng/L) in the NAA treatment wines. The concentration found in the wine produced from NAA-treated fruit is above the odor threshold for rotundone, which was determined to be 16 ng/L in red wine, for those panelists sensitive to the compound.[11]

## DISCUSSION

The results described above confirm previous studies showing that grape berry ripening and harvest can be delayed by the application of PGRs during the preveraison period of berry development. In this case, a considerable delay in harvest was achieved using NAA and a more modest delay when Ethrel was applied (Figure 2). On the basis of previous work,[3−6] Ethrel was applied earlier during berry development than NAA to achieve the desired, delaying effect. In a previous paper, it was shown that there is a likely explanation for the application of Ethrel (which releases ethylene) delaying ripening and harvest. It appears that ethylene can induce an increase in the level of biosynthesis and accumulation of the endogenous auxin indole-3-acetic acid (IAA) through the induction of putative IAA biosynthesis gene expression.[5] This increase in IAA concentration is a probable reason for the observed delay in ripening as the application of auxins has been shown to delay ripening.[8,9,22] NAA appears to be more efficacious than IAA in its ability to delay ripening. This may be due to it being a poor substrate for IAA-amino acid synthetases, the enzymes that inactivate IAA through conjugation,[2] thus increasing the longevity in the plant of any applied NAA. However, although it is less effective than NAA, it seems that an Ethrel-induced increase in IAA concentrations[5] could have occurred, and this was sufficient to delay the onset of ripening (Figure 2).

There were relatively few differences observed in the concentrations of headspace volatile compounds between the wines from control and NAA-treated grapes. Of the 164 compounds identified, only 25 were present in significantly different abundances (eight were higher in NAA wines, and 17 were higher in the control wines) (Table 3). In addition, the differences were mainly less than 2-fold. Four compounds, 2-heptanol, ethyl (*Z*)-3-hexenoate, ethyl (*E*)-2-hexenoate, and (*E*)-3-hexen-1-ol, have previously been found to be present at higher concentrations in control wine than in wine from ripening-delayed NAA-treated grapes, in Riesling, Shiraz, or both.[8,9] This indicates that NAA treatments affect the concentrations of some volatile compounds in a similar manner in different years and between different varieties. In contrast, levels of hexyl acetate and (*Z*)-3-hexenyl acetate, which were previously found to be at higher concentrations in wines made from NAA-treated Riesling grapes,[9] were higher in control wines in the current experiment (Table 3). Hexyl acetate was also at higher concentrations in NAA Shiraz wines compared to controls in a previous study.[8] These results indicate that NAA treatment and the subsequent delay in ripening significantly alter the abundances of relatively few of the measured volatile compounds. The changes in accumulation of some compounds in response to treatment with NAA seem to be consistent, but the concentrations of other compounds must be under more complex control as they vary independently.

Some compounds derived from the lipoxygenase pathway [1-hexanol, (*E*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol, and the esters hexyl acetate, ethyl (*Z*)-3-hexenoate, ethyl (*E*)-2-hexenoate, and (*Z*)-3-hexenyl acetate] were lower in the wines from NAA-treated fruit than in wines from control fruit (Table 3). The descriptors of all of these compounds are generally "green" for the alcohols and "green/fruity/apple" for the esters. However, green characters were not found to be significantly different among the wines. It is possible that these compounds are influencing the perception of "dark fruit" versus "red confection" in the wines; that is, larger amounts in the control wines make the fruit characteristics more "fresh" and "red" compared to those created by the smaller amounts in the wines from the NAA treatment where the fruit characters are described more as "cooked fruit" or "dark".

The increase in "pepper" aroma and flavor of the wine made from NAA-treated fruit was particularly noteworthy as it was observed during winemaking and was clearly distinguishable during the sensory testing (Table 2). The molecule responsible for "peppery" character in grapes has been identified as rotundone,[11] and its presence appears to be associated with the "pepper" aroma and flavor detected in the sensory analysis of the wines in this study. Although there were higher concentrations of rotundone in the wines made from Ethrel-treated grapes than in the control, the concentrations were not above the odor threshold in red wine,[11] and there was no significant difference in "pepper" character detected between the wines from control and Ethrel-treated grapes. The large increase in the level of rotundone in wine from NAA-treated grapes was detected sensorially (Table 3). Rotundone concentrations have been shown to vary considerably within a vineyard.[14] In the experiment described here, the sample replication in the field was maintained throughout winemaking, sensory analysis, and metabolite analysis, so the effects of any field variation can be tested by statistical analysis using ANOVA. While the effect of treatment on rotundone concentration was significant ($p = 0.002$), neither the effect of row ($p = 0.321$) nor the interaction of treatment and row ($p = 0.164$) was significant at the 0.05 level.

There are a number of possible explanations for the increased concentration of rotundone in wines from Ethrel-treated and, in particular, NAA-treated grapes. We have no evidence to indicate the mechanism involved in the increase in the concentration of rotundone, i.e., via an increased level of biosynthesis, a decreased level of catabolism, or a combination of both. As described above, it is feasible that auxins are the common factor in both NAA and Ethrel treatments because of the interaction of auxins and ethylene.[5,6] This may mean that there is a direct effect of auxins on rotundone metabolism that results in a sustained increase in its concentration and that the effect of NAA is greater than that for IAA because of the greater persistence of NAA in berries.

An alternative explanation is that the increased concentrations of rotundone, especially in wines made from NAA-treated grapes, were due to the delay in veraison. Certainly, the longer the delay in veraison, and therefore harvest, the greater the accumulation of rotundone. The increase in the level of rotundone could be caused by a lengthening of the period before veraison caused by the treatments. As rotundone appears to accumulate only after veraison,[13] an increase in the level of rotundone that was caused by a delay in ripening would have to result from an increase in the number of precursor molecules to rotundone during this period. The precursor for rotundone has been identified as α-guaiene, which can be converted to rotundone through oxidation.[17] Another possibility is that the delay in veraison means that ripening is occurring under different climatic conditions and that these conditions are more


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favorable for rotundone accumulation. For example, some suggestions have been made that lower temperatures may result in increased pepper character (rotundone) accumulation.[10,13,14] There was no obvious change in the pattern of minimum and maximum temperatures during the period when rotundone accumulated (Figure S1 of the Supporting Information). Therefore, any effect of temperature on rotundone accumulation is quite subtle, or there are other factors that have a significant impact. One other parameter that differed between the control and NAA-treated fruit in particular was the length of time from veraison to harvest that was longer by approximately 8 days for NAA-treated fruit than for control fruit. As rotundone can be formed via the oxidation of $\alpha$-guaiene,[17] it could be that the longer hang time of the NAA-treated fruit after harvest may have resulted in higher rotundone concentrations. Along with the increase in rotundone concentration, the concentrations of two other sesquiterpenes [$\alpha$-calacorene and cadalene (Table 3)] were higher in wines from NAA-treated fruit, indicating that there may be a more general effect of the treatment on sesquiterpene metabolism.

Sensory analysis showed that although the wines from Ethrel-treated berries were quite similar to those from control berries there was a considerable difference between control and NAA wines as nine attributes were significantly different. The difference in rotundone concentrations can explain the differences observed in "pepper" aroma and flavor, but the other attributes are unlikely to be related to rotundone. Some of these differences could be related to changes in the levels of the volatile compounds measured (see above), but there may be other, undetected differences that influence aroma and flavor. Whatever the identity of the compounds, the difference in flavor and aroma is unlikely to be due to differences in sugar levels as there was no significant difference between the TSS values at harvest as determined by ANOVA.

In summary, both Ethrel and NAA treatments delayed the onset of berry ripening and harvest. NAA delayed ripening to a greater extent than Ethrel. Similarly, both treatments significantly altered wine volatile metabolite levels, but greater differences were induced by NAA treatment. NAA treatment, and to a lesser extent Ethrel treatment, also altered wine sensory character. Wines from NAA-treated fruit were notable for having higher scores for "pepper" aroma and flavor. Analysis by GC−MS showed that these wines had concentrations of rotundone above the sensory limit. The reason for an increased level of rotundone in the NAA wines is unknown but could arise from a direct effect of NAA on rotundone accumulation or be a result of the altered timing of the ripening phase affecting sesquiterpene metabolism.

## ■ ASSOCIATED CONTENT

### Supporting Information

Graph of vineyard minimum and maximum air temperatures during the period of rotundone accumulation (Figure S1), composition of sensory reference standards used to define aroma (Table S2), and sensory attributes found to be not significantly different among the wines through descriptive analysis (Table S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

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## ■ ABBREVIATIONS USED

ANOVA, analysis of variance; DA, descriptive analysis; GC, gas chromatography; GC−MS, gas chromatography−mass spectrometry; IAA, indole-3-acetic acid; MS, mass spectrometry; NAA, 1-naphthaleneacetic acid; NMR, nuclear magnetic resonance; SIM, selected ion monitoring; SPME−GC−MS, solid phase microextraction−gas chromatography−mass spectrometry; TSS, total soluble solids

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