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Hydroxycinnamic Acid Ethyl Esters as Precursors to Ethylphenols in Wine

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ABSTRACT: A method for determining ethyl coumarate and ethyl ferulate in wine using GC-MS with deuterium-labeled analogues has been developed and used to measure the evolution of these two esters during the production of two commercial monovarietal red wines, cv. Grenache and Shiraz. During fermentation, the concentration of ethyl coumarate rose from low levels to 0.4 mg/L in Grenache and 1.6 mg/L in Shiraz wines. These concentrations then increased further during barrel aging to 1.4 and 3.6 mg/L, respectively. The concentration of ethyl ferulate was much lower, reaching a maximum of only 0.09 mg/L. Conversion of ethyl coumarate and ethyl ferulate to their corresponding ethylphenols was observed during fermentations of a synthetic medium with two strains of *Dekkera bruxellensis* (AWRI 1499 and AWRI 1608), while a third (strain AWRI 1613) produced no ethylphenols at all from these precursors. Strains AWRI 1499 and 1608 produced 4-ethylphenol from ethyl coumarate in 68% and 57% yields, respectively. The corresponding yields of 4-ethylguaiacol from ethyl ferulate were much lower, 7% and 3%. Monitoring of ethyl coumarate and ethyl ferulate concentration during the *Dekkera* fermentations showed that the selectivity for ethylphenol production according to yeast strain and the precursor was principally a result of variation in esterase activity. Consequently, ethyl coumarate can be considered to be a significant precursor to 4-ethylphenol in wines affected by these two strains of *Brettanomyces/Dekkera* yeast, while ethyl ferulate is not an important precursor to 4-ethylguaiacol.

KEYWORDS: *Brettanomyces, Dekkera, yeast, fermentation, ethyl coumarate, ethyl ferulate, 4-ethylphenol, 4-ethylguaiacol*

INTRODUCTION

Although the wine industry has largely moved past the fortuitous fermentation of grape juice by indigenous yeast, and into a world of more controlled and predictable fermentations with cultured or purchased yeasts,[1,2] undesired wine modification by microorganisms still plays a role in determining wine quality.[3] In particular, the yeast species *Dekkera* (*Brettanomyces*) *bruxellensis* has been consistently problematic in wine production throughout the world,[4] causing significant economic loss.[5,6]

Of the off-flavor compounds produced by *D. bruxellensis*, those of greatest interest are 4-ethylphenol (**1**) and 4-ethylguaiacol (**2**). These compounds, their presence in wine, and their link to *D. bruxellensis* have been extensively researched over the past 25 years,[7−17] and under oenological conditions, their evolution is attributed almost exclusively to *D. bruxellensis*.[10,14]

The first studies into the sensory impact of these volatile phenols in wine found that while 4-ethylphenol had an aroma detection threshold in a red wine of 605 μg/L and a rejection threshold of 620 μg/L, the amount of 4-ethylguaiacol needed to affect the aroma of wine was much less, being detected at 110 μg/L and the wine being rejected at 140 μg/L.[11] However, the amounts of these compounds found in wine differ greatly and, to some extent, can depend on wine variety. A survey of 61 Australian monovarietal red wines found that the ratio of 4-ethylphenol/4-ethylguaiacol varied from 10:1 in Cabernet Sauvignon to 3.5:1 in Pinot Noir and with an average ratio of approximately 8:1,[7] presumably reflecting the relative amounts of precursors present in the grape.[11] Differences in yeast nutrients, winemaking practices, *D. bruxellensis* strains, temperature, and use of oak all contribute to altering the concentration of ethylphenols.[17] When present in a red wine in a ratio of 4-ethylphenol/4-ethylguaiacol = 10:1, an aroma detection threshold for the mixture of 369 μg/L and a rejection threshold of 426 μg/L were determined.[11] In the survey of Australian red wines, approximately 60% contained these phenols above these rejection thresholds.[7]

The ethylphenols are formed via two enzymatic activities present in *D. bruxellensis* that act on *p*-coumaric acid (**3**) and ferulic acid (**4**). The first of these is a decarboxylase which converts the two hydroxycinnamic acids (**3** and **4**) into 4-vinylphenol (**5**) and 4-vinylguaiacol (**6**), respectively (Figure 1).[18] The second activity is a vinyl reductase which reduces the double bond forming the ethyl analogues (**1** and **2**).[18,19] Although many other wine microorganisms possess a hydroxycinnamic acid decarboxylase and a few also have limited vinyl reductase activity, only *Brettanomyces/Dekkera* yeasts are able to produce high concentrations of ethylphenols under oenological conditions.[10,11,14,20−23]

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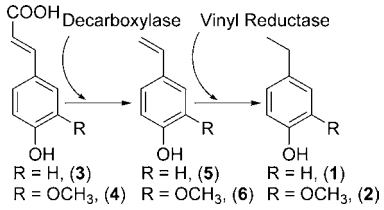

Figure 1. Formation of ethylphenols from hydroxycinnamic acids by *Brettanomyces/Dekkera* yeasts.

Apart from limiting *Dekkera* growth, one way of avoiding ethylphenol production is to minimize the concentration of precursors in the wine.[8] A study of free run juice found the hydroxycinnamoyl tartrate esters in concentrations well in excess of the hydroxycinnamic acids.[24] A common oenological technique is the addition of enzyme preparations during maceration to aid in the release of phenolic compounds from the grape berries. However, these preparations have been shown to be relatively effective in releasing free hydroxycinnamic acids from their esterified forms, which then leaves these available for conversion to the associated volatile phenols. It has been recommended that enzyme preparations possessing cinnamoyl esterase not be used in winemaking as it increases the chance of spoilage by volatile phenols.[8,25]

While various hydroxycinnamoyl derivatives have been identified in grapes, some, not present in the grape berry, are a product of the winemaking process. Somers found that ethyl coumarate (**7**) was not present in the must of commercial Chardonnay but increased throughout the course of alcoholic fermentation to be present at a concentration of 2.7 mg/L at day 31.[26]

With *D. bruxellensis* considered to possess esterase activities,[27] it is surprising that, to date, no studies have actively measured ethylphenol output when fermented in the presence of known esters of hydroxycinnamic acids. This article describes a new GC-MS method for quantifying ethyl coumarate (**7**) and ethyl ferulate (**8**) in wine (Figure 2). This method was used in

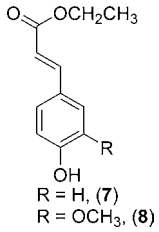

Figure 2. Ethyl coumarate and ethyl ferulate.

conjunction with a previously developed method for ethylphenols[7] to determine whether the most common strains of *D. bruxellensis* found in Australian wineries[28] could convert these ethyl esters to the corresponding phenols and whether, therefore, these esters serve as indirect sources of off-flavor in wine.

MATERIALS AND METHODS

General. Dry organic solvents were purchased and dispensed using a Puresolv solvent purification system (Innovative Technologies, Massachusetts, USA). Column chromatography was performed using Davisil 40−63 μm silica gel. Thin layer chromatography was performed using Merck silica gel 60 F_{254} alumina sheets (20 × 20 cm) and viewed under UV light. Melting points were determined using a Buchi B-540 melting point apparatus. The ^{1}H NMR spectra were acquired with a Bruker Ultrashield Plus 400 MHz Spectrometer. Spectra were of $CDCl_3$ solutions. The Shiraz and Grenache wines monitored during and after fermentation were those described by Lloyd et al.[29] 4-Ethylphenol and 4-ethylguaiacol (4-ethyl-2-methoxyphenol) were analyzed as described previously.[7]

Synthesis of Substrates and Standards. Ethyl coumarate (ethyl (2*E*)-3-(4-hydroxyphenyl)prop-2-enoate, **7**) and ethyl ferulate (ethyl (2*E*)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoate, **8**) were prepared by the method of Lang and Hansen[30] from 4-hydroxybenzaldehyde and vanillin (4-hydroxy-3-methoxybenzaldehyde), respectively. The products were purified by column chromatography on silica and had ^{1}H NMR spectra that were identical to published spectra.[31,32] (*E*)-4-Ethyl coumarate (**7**), mp. 72−73 °C (lit. mp.,[33] 73−74 °C), *m*/*z* (%) 192 (M^+, 50), 177 (3), 164 (12), 163 (6), 147 (100), 131 (3), 120 (30), 119 (24), 118 (11), 91 (20), 89 (9), 65 (13), 63 (6); (*E*)-ethyl ferulate (**8**), mp 39−41 °C (lit. mp.[34] 39 °C), *m*/*z* (%) 222 (M^+, 100), 194 (15), 177 (65), 161 (6), 150 (47), 149 (9), 148 (9), 145 (39), 135 (9), 134 (10), 133 (10), 117 (15), 105 (8), 89 (16), 78 (8), 77 (9), 63 (5), 51 (6). $^2H_2$-Ethyl coumarate and $^2H_3$-ethyl ferulate were prepared in an analogous manner from $^2H_2$-4-hydroxybenzaldehyde (**11**) and

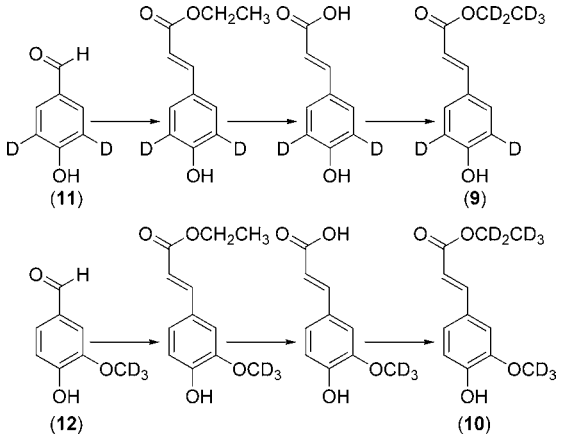

Figure 3. Preparation of deuterium labeled ethyl coumarate and ethyl ferulate.

$^2H_3$-vanillin (**12**),[35] respectively (Figure 3). The former (**11**) was formed by heating 4-hydroxybenzaldehyde (5 g) in D_2O (40 mL) and DCl (2 mL, 37% wt in D_2O) at 100−110 °C for three months. Potassium hydroxide (0.24 g) was added to a solution of $^2H_2$-ethyl coumarate (0.41 g) in aqueous ethanol (10 mL, 1:1) and the solution stirred at ambient temperature overnight. The mixture was then diluted with water and extracted with ether to remove unreacted material. The mixture was next acidified to pH 3 and the product (0.27 g) recovered with ethyl acetate. A portion of this was dried, dissolved in dry dichloromethane, and stirred with oxalyl chloride (110 μL) for 1 h. Pyridine (100 μL) and d_5-ethanol (74 μL) were then added and the reaction mixture stirred overnight. The solvent was removed in vacuo and $^2H_7$-ethyl coumarate (**9**) recovered with ethyl acetate. $^2H_8$-Ethyl ferulate (**10**) was prepared in an analogous manner. The ^{1}H NMR spectra of **9** and **10** were consistent with the spectra for the unlabeled analogues. $^2H_7$-Ethyl coumarate (**9**), *m*/*z* (%) 199 (M^+, 60), 167 (8), 166 (7), 149 (100), 148 (39), 123 (30), 122 (16), 121 (25), 120 (20), 93 (20), 66 (10), 65 (10); $^2H_8$-ethyl ferulate (**10**), *m*/*z* (%) 230 (M^+, 100), 198 (12), 180 (58), 154 (30), 152 (10), 151 (10), 145 (28), 134 (9), 133 (9), 117 (8), 89 (9).

Preparation of Samples for Analysis of Ethyl Coumarate and Ethyl Ferulate. An aliquot (25 μL) of a solution of $^2H_7$-ethyl coumarate (240 mg/L) and $^2H_8$-ethyl ferulate (257 mg/L) in ethanol was added to the wine samples (1 mL) using a glass syringe (100 μL SGE). Pentane/diethyl ether (2:1, 1 mL) was added, and the mixture was shaken briefly. A portion of the organic layer was then transferred

to a vial for GC-MS analysis. For calculating the concentration of the analytes in the wine samples, replicate standards were prepared at the same time as the hydrolysate samples, by adding the same amount of internal standard as above (25 μL) to a solution (100 μL) of ethyl coumarate and ethyl ferulate (each 100 mg/L in ethanol). This was then diluted with dichloromethane (1800 μL) and analyzed by the GC-MS method (see below) to calculate the relative response factors.

GC-MS analysis. All solvents used were of Mallinckrodt nanopure grade and verified for purity by GC-MS prior to use. Samples were analyzed with a Hewlett-Packard (HP) 6890 gas chromatograph fitted with a Gerstel MPS2 autosampler and coupled to a HP 5973N mass spectrometer. The liquid injector was operated in fast liquid injection mode with a 10 μL syringe (SGE, Australia) fitted. The gas chromatograph was fitted with an approximately 30 m × 0.25 mm I.D. J&W fused silica capillary column DB-1701, 0.25 μm film thickness. The carrier gas was helium (BOC gases, Ultra High Purity), and the flow rate was 1.2 mL/min. The oven temperature was started at 100 °C, held at this temperature for 1 min, then increased to 250 at 10 °C/min and held at this temperature for a further 20 min. The injector was held at 220 °C and the transfer line at 250 °C. The sample volume injected was 2 μL, and the splitter, at 42:1, was opened after 30 s. Fast injection was done in pulse splitless mode with an inlet pressure of 25.0 psi maintained until splitting. The glass linear (Agilent Technologies) was borosilicate glass with a plug of resilanized glass wool (2−4 mm) at the tapered end to the column. Positive ion electron impact spectra at 70 eV were recorded in the range m/z 35−350 for scan runs. For quantification of ethyl coumarate and ethyl ferulate, mass spectra were recorded in the selective ion monitoring (SIM) mode. The ions monitored in SIM runs were: m/z 123, 149, 167, and 199 for $^2H_7$-ethyl coumarate, 154, 180, 198, and 230 for $^2H_8$-ethyl ferulate, 120, 147, 164, and 192 for ethyl coumarate, and 177, 194, and 222 for ethyl ferulate. Selected fragment ions were monitored for 20 ms each. The underlined ion for each compound was the ion typically used for quantification, having the best signal-to-noise and the least interference from other wine components. The other ions were used as qualifiers.

Validation. The method was validated by a series of duplicate standard additions of unlabeled ethyl coumarate and ethyl ferulate (no addition plus each of 0.1, 0.5, 2, 3, 4, 6, 8, and 10 mg/L, $n = 2 \times 9$) to a dry white wine (Australian Chenin Blanc, 11.5% alc/vol, pH 3.40). The standard addition curves obtained were linear throughout the concentration range, with coefficients of determination (r^2) = 0.999 (ethyl coumarate) and 0.998 (ethyl ferulate). The results reported for the calibration of the methods were derived from the average of two replicate measurements for each concentration of analyte (and seven replicates for repeatability samples). The LINEST function in Microsoft Excel 2007 was used to obtain calibration function slopes and intercepts and their associated standard errors. For ethyl coumarate, the LOD was 0.014 mg/L and the LOQ was 0.045 mg/L, and for ethyl ferulate, the LOD was 0.005 mg/L, and the LOQ was 0.016 mg/L. To determine the precision of the method, seven additional replicates of the spiked wines were prepared at each of two of the concentrations (1 and 6 mg/L). The respective standard deviations for analysis at these concentrations were, for ethyl coumarate, 2.0 and 0.3%, and, for ethyl ferulate, 2.3 and 0.6%. To ensure that the accuracy of the analysis was maintained, duplicate control wines, each with and without spiked standard addition of 3 mg/L of ethyl coumarate and ethyl ferulate per liter of wine, were analyzed with every set of wine samples.

Yeast and Media. Strains of *D. bruxellensis* AWRI 1499, AWRI 1608, and AWRI 1613 were obtained from the Australian Wine Research Institute culture collection, maintained on MYPG plates (Malt Yeast Peptone Glucose). Starter cultures of single strains were prepared in YPD media (Yeast Peptone Dextrose, 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) and incubated at 28 °C with shaking (150 r.p.m.) until the cell count reached 10^8 cells/mL as determined using a hemocytometer. Fermentation experiments were performed in YNB media (Yeast Nitrogen Base, US Biologicals, supplemented with 20 g/L glucose, pH 3.5).

Fermentation Experiments. Small-scale model ferments were performed under self-anaerobiosis with occasional swirling by hand. YNB media (200 mL) were spiked with ethyl ferulate and ethyl coumarate at 10 mg/L (1 mg/mL in ethanol, 2 mL), inoculated with a single strain of *D. bruxellensis* (10^6 cells/mL), and incubated at 28 °C. Yeast growth was monitored using optical density, and the ferments concluded after stationary phase had been achieved. Control experiments were run concurrently, performed under analogous conditions without yeast inoculation. Samples (5 mL) were collected every second day and centrifuged (4000 rpm for 5 min) and the supernatant decanted from the yeast pellet and stored at −20 °C until required for analysis.

RESULTS AND DISCUSSION

Analysis of Ethyl Coumarate and Ethyl Ferulate in Wine. Deuterium-labeled analogues (**9** and **10**) of ethyl coumarate and ethyl ferulate were prepared by standard methods as shown in Figure 3. These were used as internal standards in a new GC-MS analytical method, which was applied to monitoring ethyl coumarate and ethyl ferulate formation during the production of a commercial Grenache and Shiraz wine from the beginning of fermentation to the end of barrel maturation. The analytes could be determined with good precision and accuracy over a concentration range of 0.016−10 mg/L.

For both the Grenache and Shiraz wine, the concentration of ethyl ferulate at the end of fermentation was 0.03 and 0.02 mg/L, respectively. These respective concentrations increased to 0.09 and 0.07 mg/L by the end of the maturation period, approximately 10 months after crushing. The concentrations of ethyl coumarate in the wines was much higher. In the Grenache wine, the ethyl coumarate concentration rose from 0.4 mg/L at the end of fermentation to 1.4 mg/L at the end of maturation.

The evolution of ethyl coumarate over time in the Shiraz wine is shown in Figure 4. The concentration rose sharply from just a few micrograms per liter to 1.4 mg/L by the end of fermentation and then more slowly to a high of 3.6 mg/L by the time the wine was ready for bottling.

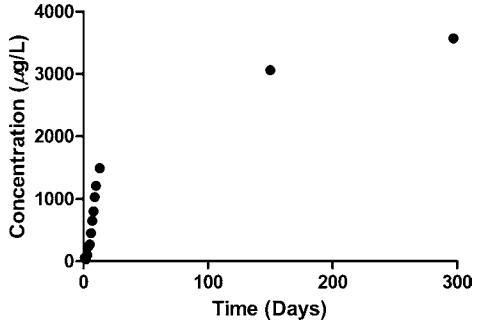

Figure 4. Evolution of ethyl coumarate in a commercial Shiraz wine during fermentation of the must and subsequent barrel maturation.

Metabolism of Ethyl Coumarate and Ethyl Ferulate by *Dekkera* Yeast. To determine whether ethyl coumarate and ethyl ferulate could, in the concentrations found in these two wines, contribute to ethylphenol formation as a result of the growth of *Brettanomyces/Dekkera* yeast, these esters were added to *D. bruxellensis* AWRI 1499 fermentations. This strain accounts for 85% of Australian isolates.[28] Yeast biomass formation peaked at around day 6 of fermentation, and the fermentations were continued for a further 3 days to maximize conversion of the ethyl esters.

Both 4-ethylphenol and 4-ethylguaiacol were formed from their respective hydroxycinnamoyl esters, while in the absence of yeast, there were no ethylphenols formed. The evolution of these ethylphenols, expressed as a percentage of the theoretical maximum that could be formed from the added hydroxycinnamates (assuming total conversion to ethylphenols), is shown in Figure 5. It is evident that the conversion of ethyl coumarate to

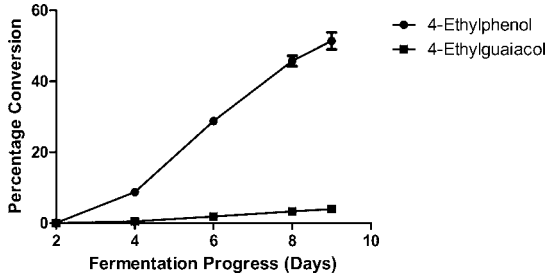

Figure 5. Evolution of 4-ethylphenol and 4-ethylguaiacol from ethyl coumarate and ethyl ferulate, respectively, during fermentation by *D. bruxellensis* strain AWRI 1499. Data are the means of three fermentations, and the error bars indicate standard deviation. Some standard deviations were close to zero and therefore not evident in the graph.

4-ethylphenol was approximately 10 times more efficient than the corresponding conversion of ethyl ferulate.

Godoy et al. purified a *p*-coumarate decarboxylase from *B. bruxellensis* and tested it for substrate specificity with *p*-coumaric acid, caffeic acid, and ferulic acid. The decarboxylase was effective toward all three substrates with an activity of 120 and 80% for caffeic and ferulic acids relative to that of *p*-coumaric acid.[18] Similar experiments were conducted by Edlin et al. who purified a hydroxycinnamate decarboxylase from *B. anomalus*. For this enzyme, the relative activities of *p*-coumaric and ferulic compared to caffeic acid were 37.5 and 31.3%, respectively.[36] These results suggested that the large difference in conversion efficiencies of hydroxycinnamic acid ethyl esters shown in Figure 5 might be due to different esterase specificities for the two substrates. The fermentation experiments were therefore repeated, and both ethylphenol formation and ethyl ester retention were monitored (Figures 6 and 7).

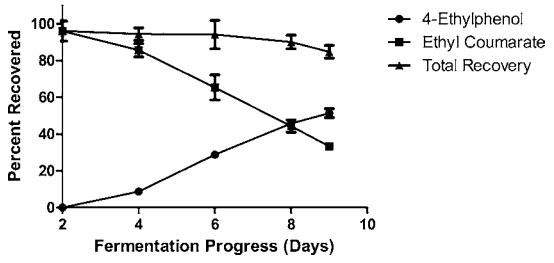

Figure 6. Recovery of 4-ethylphenol and ethyl coumarate during fermentation by *D. bruxellensis* strain AWRI 1499. Data are the means of three fermentations, and error bars indicate standard deviation. Some standard deviations were close to zero and therefore not evident in the graph.

With more ethyl ferulate remaining in the fermentations than ethyl coumarate, it was evident that the difference in the efficiency of the conversion of the hydroxycinnamic acid ethyl ester to the corresponding ethylphenol by *D. bruxellensis* strain AWRI 1499 was primarily due to preference of the yeast's ethyl

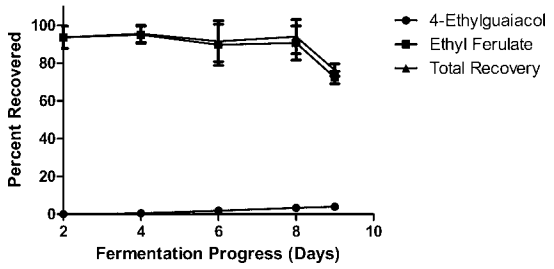

Figure 7. Recovery of 4-ethylguaiacol and ethyl ferulate during fermentation by *D. bruxellensis* strain AWRI 1499. Data are the means of three fermentations, and the error bars indicate standard deviation. Some standard deviations were close to zero and therefore not evident in the graph.

esterase activity toward ethyl coumarate. The total recovery of hydroxycinnamic ester plus ethylphenol for each substrate was around 80%, which does not include any hydroxycinnamic acid or vinylphenol intermediates that might also have been present. Fermentations were, however, conducted under anaerobic conditions that favor complete conversion of vinylphenols to ethylphenols (authors' own unpublished data). Slight losses can be expected through adsorption onto the yeast[37,38] and possibly also through the gas-lock.

To determine whether these results were strain specific, fermentations were then conducted with two other strains of *D. bruxellensis* in the presence of ethyl coumarate and ethyl ferulate. These three strains account for 98% of all of the isolates of *D. bruxellensis* recovered from Australian sources.[28] Because the earlier ferments had not been completely depleted of the ethyl hydroxycinnamates, these additional ferments, together with repeated ferments with strain AWRI 1499 were conducted for an additional three days. The percentage conversion of the substrates to their respective ethylphenols is shown in Figure 8.

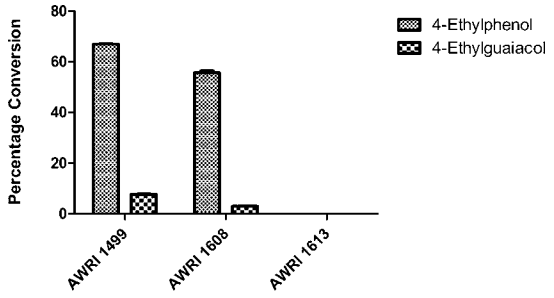

Figure 8. Percentage conversion of ethyl coumarate and ethyl ferulate to 4-ethylphenol and 4-ethylguaiacol by different strains of *D. bruxellensis*. Data are the means of three fermentations, and error bars indicate standard deviation. Some standard deviations were close to zero and therefore not evident in the graph.

With extended exposure, over 65% of the ethyl coumarate was converted to 4-ethylphenol by strain 1499. Strain 1608 performed in a similar manner, although the percentage conversions were slightly lower. Both strains displayed similar preferences for converting ethyl coumarate compared to ethyl ferulate. Strain 1613, in contrast, produced no detectable ethylphenols, a result confirmed when the experiment was repeated at a later date (data not shown). Analysis for ethyl coumarate and ethyl ferulate confirmed that both were still present at their initial concentrations in fermentations with

AWRI 1613 (data not shown). These results suggest that *D. bruxellensis* AWRI 1613 was unable to take up, or convert, hydroxycinnamate ethyl esters into free acids, rather than being unable to convert free acids into ethylphenols.

This work confirms that ethyl coumarate has the potential to contribute a high concentration of 4-ethylphenol to red wines matured in barrels when *Dekkera* growth takes place. In contrast, given the significantly lower concentration of ethyl ferulate in the wines that we have examined, this ester is unlikely to contribute significant concentrations of 4-ethylguaiacol to red wines. The greater production of 4-ethylphenol compared to 4-ethylguaiacol in wine is well documented,[7,11] and this has been attributed to the relative amounts of precursors present in the grapes. However, these results show that this ratio will be defined not only by the relative amounts of free hydroxycinnamic acids present in the berry or released by enzyme preparations during maceration but also by the amounts of ethyl coumarate produced during vinification and maturation, and the greater production of 4-ethylphenol could in fact be accentuated by the presence of this ethyl ester.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

GC-MS, gas chromatography−mass spectrometry; AWRI, Australian Wine Research Institute; NMR, nuclear magnetic resonance

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