Effect of Wine-Making Practices on the Concentrations of Fenarimol and Penconazole in Rosé Wines

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(MS# 96-320: Received 12 November 1996/Accepted 28 January 1997)

ABSTRACT

The changes in and influence of the anti-powdery-mildew fungicides fenarimol and penconazole were studied in the production and quality of rosé wines made with Monastrell grapes grown in the Jumilla wine-producing region in SE Spain. Fungicide concentrations were estimated by gas-liquid chromatography with electron-capture detection. Fermentation was retarded more by penconazole than by fenarimol; in both cases, the slowdown was directly proportional to fungicide concentration. However, the mature wine contained normal concentrations of residual sugars; other enological parameters (pH, volatile acidity, intensity of color and hue) were not significantly affected. Thirty-four days after the start of the experiment, 67% of fenarimol and 52% of penconazole, with respect to the smaller amount initially added (1 mg/liter), were found in the finished wine. The calculated half-life times were 45 and 59 days for penconazole and fenarimol respectively. Different wine-making techniques (racking, clarification, and filtration) had no decisive influence on the removal of fungicide residues from the must, although they eliminated slightly more penconazole than fenarimol.

Key words: Fenarimol, penconazole, must, residues, wine, wine-making, fungicides

The extensive areas of cropland occupied by vineyards (17% of the world total, 33% of the European Union total), together with climatological characteristics in some Spanish wine-producing regions, produce habitats that favor the growth of many different pests. Of the major pests and diseases, powdery mildew causes considerable economic losses in some areas. This fungal disease, caused by Uncinula necator (Schwein.) Burril, is widespread throughout the world, and is adapted to dry environments (9, 17).

Vine growers need to protect their crops with pesticides, which can contaminate the wine obtained from treated berries. During the first steps in the wine-producing process (i.e., crushing, draining, and pressing), pesticides in or on the grape berries can pass into the must or may remain present in the wine, depending on the production process used (7, 8, 10). Part of the residues in the must may remain for variable periods in the wine, and consumers may reject products they suspect to be contaminated. However, neither the European Union or individual wine-producing countries have established maximal residue limits (the maximum amount of a residue that is permitted in or on a food) for wines. Wine-making often reduces the amount of pesticide residues; the stages of racking and clarification of the must are apparently especially effective in removing these residues (1, 2, 11).

Pesticide residues in grape berries can have a number of undesirable effects on wine. Because of their chemical nature, they can themselves spoil the bouquet and flavor of musts and wines; indirectly, they can modify the action of microbial flora (15, 16, 18). Because the must is a complex medium, pesticide residues can undergo further transformation into entirely different products (4), and can in some cases alter the organoleptic properties of wines (3, 21).

The present study was designed to analyze changes in the concentration of fenarimol (2,4'-dichloro-α-[pyrimidin-5-yl] benzhydryl alcohol) and penconazole (1-{2,4-dichloro-β-propylphenethyl-1H-1,2,4-triazole), two anti-powdery-mildew fungicides that are widely used in the Jumilla wine-producing region (SE Spain). The purposes of this research were to determine the influence of the fungicides on fermentation, to measure removal of residues, and to determine the suitability of the wine for consumption.

MATERIALS AND METHODS

Must and wine production

The variety of grape used in all experiments was Monastrell, the major variety used to produce Jumilla wines in SE Spain. One hundred vines from a 1.5-ha plot were sampled. When the fruit was delivered to the winery it was pressed with a drum press, and stems were removed at this time to avoid giving the must woody flavors. The must was placed in a static drainer to obtain the free-run must, which was used in all experiments. No maceration step was used, and the color of the must was that given only by the period elapsed between pressing and collection of the free-run must, considered of higher quality than pressed must. Sulfites were added to the must at 150 mg/liter to prevent the growth of undesired microorganisms; no other product that might have affected the results was added. Then 10-liter amounts were placed in 15-liter capacity glass flasks,
and one of the two fungicides (fenarimol, 99.5% and penconazole, 96.1% purity were obtained from DowElanco and Ciba-Geigy, respectively) was added; one control flask was filled with must, but no fungicide was added. Before the start of the experiment, the musts were first checked to rule out the presence of either of the compounds. The initial concentration of fungicides added were fenarimol, 1.05, 6.05, and 19.81 mg/liter and penconazole, 0.9, 6.15, and 20.88 mg/liter. Two flasks were prepared with each concentration of the fungicides.

During the first 13 days of fermentation the flasks were shaken daily to stimulate yeast activity. After 13 days the musts were racked, and the residual concentrations of fungicides in the lees was determined.

To study the effects of clarification on the removal of residues from the must, flasks were left to ferment for a further 22 days; when there were no further changes in density (end of fermentation), 0.4 g of bentonite was added per liter of sample. The flasks were shaken for 1 h and left to settle for 3 days, after which clarified wine was decanted for analysis. Before it was added to the must, the bentonite was characterized by X-ray diffraction with a Philips apparatus equipped with a vertical goniometer and direct reading spectrophotometer. Specific surface area determined with the EGME (ethylene glycol monoethyl ether) method (5) was 625 m²/g.

The samples were finally filtered through a cellulose plate to obtain technically finished wine.

Enological parameters

Density was recorded daily using a Proton densimeter. Temperature, density, pH (at 25°C), total acidity, intensity of color and hue were recorded on days 0, 3, 10, and 17 of the experiment. Total acidity of the must was measured with 0.1 N NaOH up to pH 7. Intensity of color was determined as the sum of absorbances at 420 and 520 nm. Hue was characterized as the A₂₇₀/A₅₂₀ ratio. All samples were centrifuged at 4,000 × g for 10 min before the parameters were assessed.

Must and wine extraction

We used different methods of extraction of fungicides for musts and lees. For musts, 50 ml was placed in a separatory funnel and extracted by vigorously shaking the funnel for 2 min with 50 ml of hexane first, and then with 50 ml of ethyl acetate. The two separated organic phases were collected with anhydrous Na₂SO₄, and the solvent was removed by rotary vacuum evaporation. The residue was dissolved in 10 ml of isooctane plus toluene (1:1, vol/vol).

For lees, 75 ml of a mixture of acetone plus hexane (1:1, vol/vol) was added to 50 g of sample; the mixture was shaken mechanically for 1 h, and then centrifuged at 8,000 × g and filtered under a vacuum with a no. 4 porous-plate funnel. This crude extract was mixed with 50 ml of ethyl acetate twice, and the organic phases were dried, evaporated, and redissolved in 10 ml of isooctane plus toluene (1:1, vol/vol). Because the components of the must itself showed no interference with the fungicides, purification of the extracts was not necessary.

Recovery of fungicides

To determine percent recovery, musts and lees were spiked with concentrations of pesticides equivalent to the lower doses tested in this study. The results of recovery analysis were compared with those in standard dilutions used to fortify the musts. Recovery was tested in 5 samples of must (50 ml) and 5 samples of lees (50 g) that were spiked with 2.42 and 86.5 μg of penconazole (equivalent to 0.048 and 1.73 ppm respectively) and 2.52 and 90 μg of fenarimol (equivalent to 0.05 and 1.8 ppm). After the evaporation of the spiking solvent (ethanol), the samples were processed in the way indicated previously.

Gas chromatography

The gas chromatograph was a Perkin-Elmer Autosystem equipped with split/splittless capillary injector and electron-capture detector (ECD), in combination with a PE Nelson 1020 integrator. The operating temperatures were: injection port 250°C, detector 350°C, column oven, see GC columns below. The carrier gas was N₂ at 117,215 Pa. A fused silica capillary column (SPB-5) packed with 5% diphenyl, 94% dimethyl, 1% vinyl polysiloxane (slightly polar), 30 m by 0.32 mm i.d. and film thickness 0.25 μm was used (Supelco Inc.). The temperature program was: initial 90°C, hold 1 min, programming rate 30°C/min (90 to 270°C), hold 8 min at 270°C. Standard dilutions (1 μl) were injected into the chromatography apparatus, and the compounds were identified on the basis of their retention times: 8.89 min for penconazole and 13.83 min for fenarimol. An external standard containing both fungicides was used to quantify the compounds on the basis of the area under the chromatographic peak. These dilutions were also used to calculate regression equations, correlation coefficients, linearity of the detector response, and detection limits. All solvents utilized in this study were pesticide grade (SDS, France).

Statistics

Descriptive statistics and linear regression analyses used to determine the relationship between fungicide concentration and time were obtained using BMDP New System, Version 1.0 for Windows (BMDP Statistical Software, Inc., Los Angeles, CA).

RESULTS AND DISCUSSION

Analytical determinations of fungicides

The regression lines showed that the detector response was linear across the range of concentrations we studied (0.05 to 6.0 μg/ml). Linearity was confirmed by the correlation coefficients obtained, 0.9957 and 0.9986 for penconazole and fenarimol respectively.

The detection limits were found by assuming that the area below the peak on the chromatogram was at least three times the value of the background noise. This gave a detection limit of 0.02 ng/μl for fenarimol and 0.019 ng/μl for penconazole.

Table 1 shows the fungicide recoveries obtained. Recovery was greater than 90% in all assays with the lowest

| Fungicide | Amount added (ppm) | Sample | Recovery (mean ± SD, n = 5) | SEM | CV (%)
|-----------|--------------------|--------|-----------------------------|-----|------
| Fenarimol | 0.05               | M      | 93.2 ± 3.49                 | 1.56| 3.75 |
|           |                    | L      | 97.6 ± 2.70                 | 1.21| 2.77 |
|           | 1.0                | M      | 72 ± 5.70                   | 2.55| 7.92 |
|           |                    | L      | 75.4 ± 4.61                 | 2.06| 6.12 |
| Penconazole | 0.048             | M      | 90.4 ± 2.41                 | 1.08| 2.66 |
|           |                    | L      | 101.4 ± 3.51                | 1.57| 3.46 |
|           | 1.73               | M      | 79.2 ± 4.97                 | 2.22| 6.28 |
|           |                    | L      | 92.8 ± 2.59                 | 1.16| 2.79 |

* M, must and L, lees.
concentration of fungicide (0.05 ppm); in both musts and lees, mean recovery was higher than 90%, and coefficients of variability were within an acceptable range of 2 to 4%. When the dose of pesticide was 1.8 ppm, recovery was satisfactory with penconazole, and below 80% with fenarimol.

Mean recovery values were used to calculate the theoretical limits of sensitivity (TLS) of the analytical method as the minimum concentration of fungicide (in mg/kg) that could be detected accurately. The detection limit found for each compound was calculated with the formula:

\[ TLS = V_e \cdot \frac{DL}{V_i} \cdot W \]

where \( V_e \) is the volume of extract (in ml), \( DL \) is the limit of detection (in ng), \( V_i \) is the volume of extract injected for chromatography (in µl) and \( W \) is the mass (g) of the initial sample.

The TLS for fenarimol was 0.004 mg/kg, and that for penconazole was 0.0038 mg/kg. Multiplying the TLS by the overall performance of the method yielded (82.6 and 84.8, respectively) the real limit of sensitivity (RLS), which was 0.0033 mg/kg for fenarimol and 0.0032 mg/kg for penconazole. These values demonstrate the suitability of the analytical method for measuring the two compounds of interest.

Changes in fungicide residues

The final concentration of fungicide residues was determined in the samples to which 1.05 mg of fenarimol per liter and 0.9 mg of penconazole per liter had been added. The highest concentrations were used only to study their influence on the must fermentation. Figure 1 illustrates the changes in concentrations of fungicide residues during the 35-day experimental period. After 34 days, 33% of the fenarimol and 48% of the penconazole had been lost.

By plotting the changes in the concentrations of residues as an exponential function of time, we obtained decline curves (6), which are defined by the equation:

\[ R_t = R_0 \cdot e^{-Kt} \]

where \( R_t \) is the concentration of residues at time \( t \), \( R_0 \) is the theoretical initial concentration (in mg/kg) of residues, \( K \) is the rate constant of the process, and \( t \) is the time elapsed from the moment of addition.

To facilitate our analysis, we used a semilogarithmic linear regression (20) to transform the decline curve into a straight line. The resulting equation was:

\[ \ln R_t = \ln R_0 - Kt \]

where \( R_0 \) and \( K \) are constant and denote the y-intercept and the slope of the dissipation lines, respectively. \( R_t \) is the concentration of penconazole and/or fenarimol in the must and \( t \) is the post-application time. Table 2 shows the rate constants for the process. To appraise the correlation coefficient \( (r) \) obtained, a test quantity was realized, which indicates whether there is a correlation between residue and time (20). As the quantity \( (D) \), is, in both cases, greater than 0, the correlation between residue and time is confirmed. The data presented in this table show that the dissipation rate for fenarimol and penconazole is 0.0117 and 0.0153 days\(^{-1}\), i.e., at the end of 34 days, 0.41 and 0.51 mg/liter of fenarimol and penconazole, respectively, would have disappeared. The relationship between both constant is \( K_p/K_f = 0.76 \), which indicates a greater dissipation rate of penconazole in the must.

Also, from the data shown in Table 2 a theoretical initial residue \( (R_0) \) can be calculated of 0.77 and 0.63 mg/kg (95% confidence interval, 0.64 to 0.92 and 0.49 to 0.79 mg/kg) for fenarimol and penconazole, respectively.

From the equation given above, the half-life \( (t_{1/2}) \), defined as the time necessary for the initial amount of residue to be reduced by half, can be calculated with the formula:

\[ t_{1/2} = \ln 2/K \]

Using the rate constants found for fenarimol and penconazole we obtained half-lives of 59.2 days for the former, and 45.3 days for the latter.

In some cases, two statistical fits corresponding to two consecutive processes with first-order kinetics were invoked to explain the decline kinetics. In other words, two lines with different slopes, the first larger than the second, were obtained (14). In our case, the rate constant for the first stage (0 to 13 days) was higher than for the second stage (3 to 34 days), suggesting that degradation took place in two stages: an initial rapid phase lasting approximately until day 13, and a slower degradation, while the density of the must stabilized. This hypothesis is supported by the relationship between the rate constants, which were \( K_2/K_1 = 4.28 \) for fenarimol and \( K_2/K_1 = 4.45 \) for penconazole.

After 80 days, the concentrations of residues in the wines were 0.43 mg/liter of fenarimol, approximately double the maximal residue limit (MRL) of 0.2 mg/kg of grapes recommended by the Alimentarius Codex of the Food and Agriculture Organization (FAO) of the United Nations, and 0.19 mg/kg of penconazole, quadruple the recommended MRL of 0.05 mg/kg of grapes. From the equations depicting the logarithmic dissipation plots, and establishing these MRLs as desirable, we can calculate that 115 and 165 days would be enough time for the degradation of fenarimol and

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fenarimol</th>
<th>Penconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n )</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Min</td>
<td>-0.59</td>
<td>-0.92</td>
</tr>
<tr>
<td>Max</td>
<td>-0.10</td>
<td>-0.26</td>
</tr>
<tr>
<td>( r )</td>
<td>-0.8357</td>
<td>-0.8386</td>
</tr>
<tr>
<td>( D )</td>
<td>0.0258</td>
<td>0.0261</td>
</tr>
<tr>
<td>( S_0 )</td>
<td>0.1150</td>
<td>0.1505</td>
</tr>
<tr>
<td>y intercept ± SE</td>
<td>-0.2615 ± 0.065</td>
<td>-0.4669 ± 0.015</td>
</tr>
<tr>
<td>K (slope) ± SE</td>
<td>-0.117 ± 0.004</td>
<td>-0.0153 ± 0.005</td>
</tr>
</tbody>
</table>

FIGURE 1. Changes with time in the concentration of fenarimol and penconazole remaining in must.
penconazole, respectively. Therefore, the safety intervals recommended for both fungicides must be strictly adhered to before the grapes are harvested, as their rate of breakdown in rosé must and wine is slow.

**Influence of enological technology**

The concentrations of residues in the lees after racking were 0.7 mg/kg for fenarimol and 0.99 mg/kg for penconazole. Because 1 liter of wine produced approximately 25 g of lees, the total amounts retained were calculated as 0.18 mg of fenarimol and 0.25 mg of penconazole, values that correspond to 1.7% and 2.7% of the amount initially added to the must. These low values were not surprising, given that the solubility in water of both fenarimol (13.7 mg/liter) and penconazole (70 mg/liter) is higher than the initial concentration of the fungicides in the musts analyzed here.

The concentrations of residues after clarification and filtration to remove residues are given in Table 3. Neither of these two steps decreased the concentration of penconazole to any great extent. However, clarification decreased the concentration of penconazole by 13%, and filtration led to a further decrease of 12%.

**Enological parameters**

Figures 2 and 3 illustrate the fermentation rate. After fermentation, in all cases, the wines were normal in terms of residual sugar content. However, in musts treated with fenarimol, the change in density during days 3 to 5 (exponential growth phase of yeasts) and days 8 to 9 (beginning of the stabilization of density) was delayed when higher concentrations of fungicide were added. The second delay occurred at a critical moment for the development of secondary aromatic compounds during alcoholic fermentation, and may therefore affect the wine’s organoleptic characteristics. A detailed analysis of the synthesis and evolution of these compounds and also the types and numbers of yeasts and bacteria should be undertaken in subsequent studies.

Penconazole delayed the change in density more than fenarimol did. Both fungicides act by inhibiting the synthesis of sterols, which are necessary for the anaerobic growth of yeast cells. Sterols are located in the cell membrane, which is responsible for chemical exchanges between the yeast and the external medium. Accordingly, the fungicides may have had no effect at the beginning of fermentation when the must is in contact with the air. However, once a reducing anaerobic atmosphere is established, sterol synthesis is inhibited, and the rate of fermentation is slowed.

**TABLE 3. Concentrations of fungicide residues after clarification and filtration of musts**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fenarimol (Mean (n = 3) residue, mg/liter)</th>
<th>Penconazole (Mean (n = 3) residue, mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before clarification</td>
<td>0.55</td>
<td>0.40</td>
</tr>
<tr>
<td>Clarified</td>
<td>0.57 (104)</td>
<td>0.35 (87)</td>
</tr>
<tr>
<td>Clarified and filtered</td>
<td>0.52 (95)</td>
<td>0.30 (75)</td>
</tr>
</tbody>
</table>

In some cases, volatile acidity increases and residual unfermented sugars remain, as was found in assays with the anti-powdery-mildew fungicide triadimefon (12, 13, 19). The delay in fermentation, while not significant in the present pilot study with small volumes of must, may affect the quality of wines produced on an industrial scale (10,000 liters or greater), in which fermentation is much slower.

Table 4 shows the values of the enological parameters obtained. In all experiments we found a moderate increase in pH, from 3.4 to 3.6. This was mainly because of the degradation of malic acid and consequent loss of acid groups, together with the precipitation of potassium bitartrate, which became insoluble with increasing concentrations of alcohol in the medium as fermentation proceeded. The final pH values were considered normal, and neither of the fungicides at any concentration tested here was considered to interfere with this parameter. Total acidity increased initially, then stabilized, and subsequently decreased in some experiments. The final values for total acidity were considered within the normal limits of between 60 meq/liter (4.32 g of tartaric acid per liter) and 65 meq/liter (4.67 g of tartaric acid per liter) set down by the Jumilla wine-growers association (“apellation d’origine”) for wines classified as Jumilla Monastrell.

In all samples, intensity of color and hue underwent a similar sequence of changes regardless of the dose of fungicide added. We observed an initial decrease in color as a result of decoloration of anthocyanins in the reducing atmosphere. This was a characteristic finding during the first days of fermentation, when large amounts of carbon dioxide are released, and little or no oxygen is present. Once the rate...
of fermentation declined, anthocyanins recovered their initial structure, and the intensity of color increased. Intensity at the end of the fermentation period was similar to that at the start, as the wine tested was a rosé, and no maceration of the skins was involved. The evolution of hue was also normal. At the start of fermentation, monomeric anthocyanins predominated, so that the polymer/monomer ratio was low. The ratio increased as polymerization proceeded, and the final hue was considered within normal limits.

These findings show that fenarimol and penconazole did not have significant effects on the wine obtained at the end of fermentation. The process that was most clearly affected was transformation of the sugars, which was slowed when higher concentrations of fungicide, especially penconazole, were added.

ACKNOWLEDGMENTS

We thank the Bodega Cooperativa San Isidro of Jumilla in Murcia (Spain) for their help with this research, and Ms. Karen Shashok for translating the original manuscript into English.

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