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Fate of Pesticides during the Winemaking Process in Relation to Malolactic Fermentation

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The effect of red wine malolactic fermentation on the fate of seven fungicides (carbendazim, chlorothalonil, fenarimol, metalaxyl, oxadixyl, procymidone, and triadimenol) and three insecticides (carbaryl, chlorpyrifos, and dicofol) was investigated. After malolactic fermentation using *Oenococcus oeni*, which simulated common Australian enological conditions, the concentrations of the active compounds chlorpyrifos and dicofol were the most significantly reduced, whereas the concentrations of chlorothalonil and procymidone diminished only slightly. The effect of these pesticides on the activity of the bacteria was also studied. Dicofol had a major inhibitory effect on the catabolism of malic acid, whereas chlorothalonil, chlorpyrifos, and fenarimol had only a minor effect.

KEYWORDS: Pesticide residues; malolactic fermentation; winemaking; matrix effects

INTRODUCTION

Malolactic fermentation (MLF) is a bacterial process chiefly employed to decarboxylate L-(−)-malic acid to L-(+)-lactic acid enzymatically. This process is carried out by lactic acid bacteria (LAB), which include the genera *Lactobacillus*, *Oenococcus* (formally *Leuconostoc*), and *Pediococcus*. Of the species within these genera, *Oenococcus oeni* (*O. oeni*) is the predominant organism used in Australian winemaking (*1*).

MLF is also a deacidification step as the diacidic malic acid is converted to the monoacidic lactic acid, liberating carbon dioxide (*1*−*3*). This results in a decrease in titratable acidity and an increase in pH of the wine. This may be desirable for highly acidic wines, but for Australian wines, which tend to be made from ripe grapes with low acidity, an acid addition is usually required during the winemaking process for desirable mouthfeel. Tartaric acid is added to achieve this characteristic. Some authors report that tartaric acid is not metabolized by LAB (*1*−*3*). However, evidence for the metabolism of tartaric acid by lactic acid bacteria has been reported by Ribéreau-Gayon et al. (*4*). The increase in pH caused by MLF can also affect the intensity of color in red wine. Susceptibility to bacterial spoilage is also diminished after MLF as LAB are nutritionally fastidious, and so the resulting wine is depleted of nutrients that otherwise could support further microbiological activity (*1*, *2*). The sensory properties of the wine are altered by malolactic fermentation. Diacetyl is produced, which can give a buttery character adding complexity (*5*). A loss of fruit aroma and the reduction of varietal characters have also been reported (*1*). MLF can be inhibited by sulfur dioxide concentrations above 50 mg/L, pH values below 3.3, and ethanol concentrations above 14% (v/v) (*6*). Whereas any one of these conditions alone may not affect MLF drastically, it is usually a combination of these conditions that leads to inhibition of activity, the degree of which is strain dependent (*1*−*3*, *6*). Temperatures between 18 and 25 °C and low oxygen concentrations are preferred for MLF because LAB fall in the range of microaerophilic to facultatively anaerobic organisms (*3*).

In red wines MLF occurs often without inoculation and may proceed after inoculation. MLF occurs in only a small portion of white wines with or without inoculation. Malolactic fermentation can occur naturally or be induced by adding a bacterial starter culture. The wine is usually inoculated during or soon after the alcoholic fermentation and is monitored by the depletion of malic acid using a chromatographic or enzymatic assay (*1*−*3*).

Several studies have investigated the concentration of pesticide residues under different typical winemaking conditions (*7*−*14*), which often involve MLF. Malolactic fermentation will sometimes progress more slowly than desired. In addition to the causative conditions outlined above, some studies (*7*−*12*, *14*) have directly or indirectly investigated the effect of pesticide residues on the rate of MLF, and a few studies (*8*, *9*, *11*) have investigated the effect of this process on the concentration of pesticide residues in wine. In most cases, pesticide residues were found to have little or no effect on MLF, and very few pesticides were degraded or adsorbed by the bacteria during this process, although this appeared to be strain dependent. Cabras et al. (*8*, *9*) reported that the fermentation activity of *Leuconostoc oenos*

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Table 1. Data from Chemical Analysis of the Shiraz Wine Used in This Study

pH	3.35
titratable acidity (g/L)	7.6
free SO_2 (mg/L)	2
total SO_2[a] (mg/L)	2
glucose + fructose (g/L)	0.2
volatile acidity (g/L)	0.14
alcohol content (% v/v)	13.2
malic acid content (g/L)	3.26
pesticide content[b] (mg/L)	metalaxyl 0.013

[a] Total SO_2 is the sum of free and bound SO_2. [b] The metalaxyl present at this trace level will have little or no influence on the outcome of the results as a much higher level had no effect on MLF (**Table 3**). No other pesticides were detected when the wine was analyzed by the multiresidue assay as outlined in Ruediger et al. (*17*).

(*O. oeni*) was affected by the presence of certain pesticides. Due to the very low number of published studies in this area and because no such trials have been conducted in Australia, this study was undertaken to determine the fate of pesticides that are commonly used in Australian viticulture, under Australian winemaking conditions.

MATERIALS AND METHODS

Materials. *Wine and Bacterial Strain.* The wine used was a commercially prepared Shiraz wine, and a sample of the commercial batch was collected from a winery for use in this study. No SO_2 was added to the must prior to fermentation, and there was minimal change in SO_2 concentration during fermentation. The wine was reductively handled pre- and postfermentation. The wine was analyzed for pH and titratable acidity according to the method of Amerine and Ough (*15*), free sulfur dioxide and total SO_2 were analyzed according to the method of Rankine and Pocock (*16*), volatile acidity was analyzed by steam distillation using a modified Markham still, glucose plus fructose and malic acid were determined enzymatically, alcohol concentration was determined by near-infrared spectroscopy, and pesticide residues used in the study were analyzed according to the method of Ruediger et al. (*17*) (**Table 1**). After analysis, the wine was stored under refrigeration with no ullage and sterile filtered, as described below, prior to inoculation. The bacterial strain used was Lalvin *L. oenos* (*O. oeni*) EQ54 (Lallemand S.A.).

Chemicals. The pesticides used in this study were of analytical grade, that is, 95% or greater purity, verified by gas chromatography−mass spectrometry (GC-MS), and as defined in Ruediger et al. (*17*). Each of the 10 pesticides used in this study is from a major chemical family (see **Table 2**) as defined by the British Crop Protection Council (*18*) and were used at levels equivalent to the Australian maximum residue limit (MRL) (*19*). When a MRL set by a country importing large quantities of Australian wine is lower than the Australian MRL, then that level was also included in the study (*19*; see **Table 2**). The pesticides were selected on the basis of their common use in Australian viticulture and to ensure coverage of a wide variety of chemical families. The solvents were of pesticide grade or better, and all other reagents were of analytical grade.

Malolactic Fermentation Procedure. The wine was filtered through a glass fiber filter (Gelman Sciences, Ann Arbor, MI), then through a 0.8 μm membrane (Gelman Sciences), and finally through a sterile 0.2 μm capsule filter (Sartorius, Göttingen, Germany). Wine (500 mL) was aseptically transferred to sterile bottles, each spiked with one of the pesticides at the appropriate level (as defined in **Table 2**) and mixed thoroughly. Spiking volumes varied from 25 μL to 1.5 mL, and so the wine was not significantly diluted. A sample (50 mL) was taken for triplicate analysis, to determine the pesticide residue concentration, prior to MLF. The remaining wine was inoculated with a 4% (w/v) rehydrated *O. oeni* (EQ54) suspension (100 μL), as described by the manufacturer, to give an inoculation rate of $\sim 1 \times 10^6$ colony-forming units (CFU)/mL and mixed thoroughly. The inoculated wine was aseptically transferred into three sterile bottles and fermented at 20 °C under anaerobic conditions. The sterile bottles were fitted with a sampling port protected by a 0.45 μm filter and an air lock. Fermentation was allowed to proceed for 52 days, after which 50 mg/L SO_2 was added as potassium metabisulfite, mixed thoroughly, and allowed to settle at 2 °C for 2 days. The samples were then centrifuged at 3000 rpm for 10 min and analyzed for residues of the added pesticide. All treatments, and control samples that were spiked with solvent only to determine if the solvent would inhibit MLF, were conducted in triplicate.

Table 2. Pesticides and Levels Used in This Study

pesticide[a]	chemical family	concn (mg/L)	MRL[b]	solubility[c] (mg/L)
fungicides				
carbendazim	benzimidazole	3.0	Australian	29
		0.1	Canadian	
chlorothalonil	benzene dicarbonitrile	1.0[d]	Australian	0.81
		0.1	Canadian	
fenarimol	pyrimidinyl carbinol	0.1	Australian	13.7
metalaxyl	acylalanine	1.0	Australian	8400
oxadixyl	xylidide	2.0	Australian	3400
		0.1	Canadian	
procymidone	dicarboximide	2.0	Australian	4.5
		0.1	Canadian	
triadimenol	azole	0.5	Australian	62
		0.1	Canadian	
insecticides				
carbaryl	carbamate	5.0	Australian	120
		1.0	Japanese	
chlorpyrifos	organophosphorus	1.0	Australian	1.4
		0.1	Canadian	
dicofol[e]	organochlorine	5.0[f]	Australian	0.8
		2.0	European Union	

[a] The structures of these pesticides have been published (*17*, *18*). [b] Maximum residue limit. [c] Solubilities are in water (*18*). [d] The Australian MRL is 10 mg/L. The solubility of chlorothalonil in water is 0.81 mg/L but greater in organic solvents. A solubility test in wine, which contains between 10 and 15% ethanol, indicted that chlorothalonil was soluble above 1.0 mg/L. [e] Both the ortho,para (op) and para,para (pp) isomers were studied in the ratio of 20:80, respectively (as they occur in the commercial formulation), and quantified separately. [f] The solubility of dicofol in water is 0.8 mg/L but greater in organic solvents. A solubility test in wine, which contains between 10 and 15% ethanol, indicted that dicofol was soluble above 5.0 mg/L.

Extraction Procedure, Chromatographic Analysis, and Validation. The extraction procedure, chromatographic analysis, and validation were performed as described in Ruediger et al. (*17*), which also details the use of calibrants in matrix and analytical controls.

Statistical Analysis. Each fermentation was conducted in triplicate, and 1 in 10 extractions and analyses was duplicated. To assess whether a fermentation had a significant effect on the removal of pesticides, a comparison was made of the 95% confidence intervals (two standard errors of the mean), for the pre- and postfermentation concentrations.

RESULTS AND DISCUSSION

The chemical profile of the wine is shown in **Table 1**. The data presented can be considered to be typical for a commercially prepared red wine of this type, which has been carefully handled and which has completed primary alcoholic fermentation but has not undergone malolactic fermentation.

Table 3 reports the percentage changes of the various pesticide concentrations and percentage decrease of the malic acid concentration with respect to the appropriate control for the red wine.

MLF resulted in little or no significant reduction in pesticide concentrations except for chlorpyrifos and dicofol. Chlorpyrifos concentrations were reduced by ~70% at both concentration levels. The total dicofol concentration was reduced by >30%, with the para,para isomer being reduced by >40% and the ortho,para isomer being unaffected. This trend was observed at both concentration levels. Chlorothalonil concentration was

Table 3. Mean[a] Percent Residual Pesticide Remaining in Postmalolactic Fermentation Treatment and Mean[a] Percent Reduction of Malic Acid for a Shiraz Wine

pesticide		post-MLF (%)	% reduction of malic acid concn[b]
carbaryl	high	125[e]	91
	low	103	92
carbendazim	high	78[e]	102
	low	78	103
chlorothalonil	high	66[e]	82[e]
	low	81	84[e]
chlorpyrifos	high	27[e]	92
	low	32[e]	76[e]
dicofol op[c]	high	111	*f*
	low	107	*f*
dicofol pp[d]	high	58[e]	*f*
	low	56[e]	*f*
dicofol total	high	69[e]	6[e]
	low	66[e]	13[e]
fenarimol		187[e]	84[e]
metalaxyl		99	101
oxadixyl	high	116	96
	low	82	90
procymidone	high	92	117[e]
	low	76[e]	97
triadimenol	high	113[e]	95
	low	134[e]	99

[a] Mean of triplicate determinations. [b] Percent reduction compared to the controls taken as 100%. [c] op = ortho,para isomer. [d] pp = para,para isomer. [e] Indicates a significant difference compared to the pre-MLF concentration ($p < 0.05$). [f] Both the op and pp isomers are present in the ratio of 20:80 respectively as per commercial dicofol formulations, which were added to the wine as a combined reference standard, hence we did not study the effect of the individual purified isomers on MLF separately.

mildly reduced (35%) at the higher concentration. Some reduction in procymidone concentration was observed (25%) but only at the lower concentration. These reductions of pesticide concentrations could possibly be due to the absorption onto the bacterial cell walls, rather than chemical or biological degradation as shown by Cabras et al. (*9*, *11*), who determined the concentrations of pesticides remaining in the bacteria.

MLF was generally unaffected by the presence of pesticide residues except in the case of dicofol, where only 6−13% of the malic acid was metabolized. Dicofol can be hydrolyzed to the corresponding benzophenone and chloroform (*17*), and either the parent compound, the hydrolytic products, or a combination of all three compounds could have a detrimental effect on the bacteria. As might be expected, the higher concentration of dicofol resulted in greater inhibition of MLF. Chlorothalonil and fenarimol showed only a minor effect on the conversion of malic acid to lactic acid (82−84% metabolized), whereas chlorpyrifos at the lower concentration showed a similar effect (76% malic acid metabolized). Malolactic fermentation for the controls without carbendazim was 95% complete, whereas MLF for the controls for the other pesticides was 80% complete on the basis of the criterion that 0.1 g/L of malic acid is considered as complete. Studies on LAB inhibition by pesticides (*7*, *9*, *12*) report minimum inhibitory concentrations (MIC) for various pesticides ranging from as low as 1 to >30 mg/L. As some concentrations of the pesticides used in this study fall within this inhibitory range, the lesser extent of fermentation observed for some of the treatments can be attributed to the presence of the pesticide.

Compared to the controls, significant increases in the concentrations of carbaryl, fenarimol, and triadimenol were observed post-MLF. It has been reported that pesticide residues which contain a diverse array of functional groups are the type of analytes most susceptible to the so-called "matrix-induced chromatographic response enhancement" effect (*20*−*25*). This phenomenon can lead to recoveries of >100% [or indeed >150% (*24*)] in regard to the GC analysis of pesticides (*25*, *26*). The effect as defined by Erney et al. (*20*) is seen when improved chromatographic peak shape and intensity of the compounds of interest are observed when the analytes are injected in the presence of a complex matrix (such as a wine extract). When no matrix is present (or the matrix is simpler), then poorer peaks with lower responses result for the same susceptible compounds, even though they are present in the matrix at the same concentration. The accepted explanation (*20*, *25*, *26*) is that the matrix components protect against the active sites within the GC system; thus, there are lower levels of loss of the compounds of interest leading to better chromatographic peak shapes and higher intensities. Hydrogen bonding has also been implicated as an important factor in analyte interactions with active sites (see, e.g., refs *25* and *27*). We have taken every reasonable action to ensure that possible matrix degradation and enhancement effects are kept to a minimum, such as optimizing the GC-MS conditions and ensuring that samples are analyzed versus calibrants made up in the same matrix (i.e., wine), as detailed in Ruediger et al. (*17*).

In conclusion, malolactic fermentation using *O. oeni* resulted in little change in pesticide residue concentrations except in the case of chlorpyrifos and dicofol. In the case of dicofol, the loss was for the para,para isomer, whereas the ortho,para isomer was unaffected. The concentrations of chlorothalonil and procymidone diminished only slightly. MLF was generally unaffected by the presence of pesticide residues except in the case of dicofol, which had a major inhibitory effect at the concentrations used. Chlorothalonil, chlorpyrifos, and fenarimol appeared to have a minor inhibitory effect. The level of residual pesticides found in Australian wines has repeatedly been found to be extremely low and in full compliance with legislated MRLs in domestic and export markets. The fact that the high concentrations of most pesticides had little effect on MLF indicates that, at the concentration at which these compounds normally occur in wine (if at all), these pesticides are not responsible for the sluggish progress of MLF. In the case of dicofol, however, a substantial slowing of MLF was observed when this compound was present at high concentration. The effect of dicofol at lower concentrations remains to be tested.

ABBREVIATIONS USED

CFU, colony-forming units; GC, gas chromatography; MIC, minimum inhibitory concentration; MLF, malolactic fermentation; MRL, maximum residue limit; MS, mass spectrometry.

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