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Toxicity effects of fungicide residues on the wine-producing process

Ricardo C. Calhelha, João V. Andrade, Isabel C. Ferreira, Letícia M. Estevinho*

Mountain Research Center, Escola Superior Agrária de Bragança, Campus de Santa Apolónia, Apartado 1172, 5301-854 Bragança, Portugal

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Abstract

We report the detection of several fungicide residues (Dichlofluanid, Benomyl, Iprodione, Procymidone and Vinclozolin) in red and white bottled wines from two Portuguese wine-producing zones. Studies were done in order to evaluate the active compound transfer percentage from grapes to the final product along fermentation process. We also investigated their effects in *Saccharomyces* and non-*Saccharomyces* yeasts as well as their influence on the physical, chemical and organoleptic wine properties. All the tested fungicides had a negative effect on in vitro yeast growth, with Dichlofluanid and Benomyl being the most toxic. *Zygosaccharomyces rouxii* and *Saccharomyces cerevisiae* were the most resistant yeasts while *Rhodotorula glutinis* was the most susceptible. Microvinification experiments carried out in the presence of fungicides produced wine samples with unaltered physical, chemical and organoleptic properties, though Benomyl, a slight delay in the initiation of fermentation process was observed.

Keywords: Wine; Fungicides; Toxicity; Yeasts; Microvinification; Quality control

1. Introduction

'Brunch Rot' of grapes, caused by the fungus *Botrytis cinerea*, is a significant problem for the world-wine industry. *Plasmopara viticola* and *Unicula necator* are also common fungi encountered in viticulture pests. Although chemical fungicides have been used to combat these problems (Griffiths et al., 1998), they can be non-specific and therefore can act on organisms other than the target fungus, including other naturally occurring beneficial or pathogenic organisms (Ochiai et al., 2002). Because of their chemical nature, they may also be toxic (Radice et al., 1998; Datta and Gopal, 1999; Gray et al., 1999) and non-biodegradable. Chemical residues can build up in the soil (Athiel et al., 1995) and throughout the food chain (Radice et al., 2001). Consumers world-wide are increasingly conscious of the potential environmental and health problems (Rankin et al., 1989; Bruynzeel et al., 1995; Draper et al., 2003) associated with the build-up of toxic chemicals, particularly in food products (Mukherjee et al., 2003; Lopez and Riba, 1999).

Historically, studies on enological microbiology have centered on yeasts belonging to the genus *Saccharomyces* that are responsible for alcoholic fermentation. However, other yeasts, especially non-*Saccharomyces* yeasts present in the initial stages of fermentation process, may influence on the final organoleptic properties of the wine (Pretorius et al., 1999). These genera include *Kloeckera*, *Cryptococcus*, *Torulaspora*, *Hansieniaspora*, *Candida*, *Pichia*, *Hansenula*, *Zygosaccharomyces*, *Metschinikowia*, *Debaromyces*, *Issatchenkia* and *Rhodotorula*. Evidence exists that non-*Saccharomyces* yeasts may influence the unique enological characteristics of each wine-producing zone (Ganga and Martinez, 2004) and the presence of fungicides can affect yeasts metabolic activity.

Studies on individual determination of some pesticides in grape must and wine at residue level have been published (Cabras et al., 1983; López et al., 1989; Cabras et al., 1998; Cabras and Angioni, 2000). Each country has its own allowed maximal residue levels that

*Corresponding author. Tel.: +351 273303342; fax: +351 273325405.
E-mail address: leticia@ipb.pt (L.M. Estevinho).

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reflect the agricultural practice followed, but fungicides employment should be controlled because of its toxicity effects.

In the present work, fungicide residues were determined in red and white bottled wines for two different Portuguese wine-producing regions. The transfer percentage of each fungicide from grapes to the final product is also presented. In order to determine what impact these fungicides may have on the quality of wine produced, the current study evaluated fungicide residues effects in wine-producing process, particularly the growth of fermentation yeasts. In vitro and in vivo experiments were done to evaluate those effects for several yeasts present in fermentation process and on the physical, chemical and organoleptic properties of the resulting wine.

2. Material and methods

2.1. Materials and reagents

HPLC apparatus: a VARIAN 9050 model (1994) equipped with a UV–visible system. GC apparatus: a DANI 1000 model Gas chromatograph (2000) equipped with an ECD detector. Orbital shaker incubator: a Stuart Scientific SI50 model (2001). Autoclave: a P Selecta model (2002). UV–vis spectrophotometer: a Varian Cary 50 Scan model (1998).

Fungicides applied in grapes (trademark compounds) were obtained in a local market: Benomyl (Benlate 50% w/w; DuPont), Dichlofluanid (Euparene 50% w/w; Bayer), Iprodione (Rovral 50% w/w; Rhône-Poulenc), Procymidone (Sumisclex 50% w/w; Zaneca-Agro) and Vinclozolin (Ronilan 50% w/w; Basf). All other chemicals, including fungicides used in the in vitro assays (analytical standard compounds), were of the highest available quality, and purchased from Merck (Darmstadt, Germany).

2.2. Chromatographic determination of fungicide residues in grape must or wine samples

Extraction procedures: The organic solvent (2×50 ml, methylene chloride and petroleum ether for Benomyl and cyclohexane for Dichlofluanid, Iprodione, Procymidone and Vinclozolin) was added to the corresponding sample (50 ml) in a glass screw-capped tube, and the mixture was shaken twice for 30 min. After the phase separation stage, organic layers were collected and evaporated to dryness under reduced pressure (15 mmHg, ~40 °C). The residue was dissolved in 2 ml of methanol.

HPLC determination of Benomyl: The operating conditions were as follows: a 20×0.4 cm (i.d.), 10 μm ODS C18 column; a mixture of CH_3CN, H_2O and buffer solution (40:45:15% v/v) as mobile phase at a flow-rate of 0.8 ml/min. The buffer solution was prepared mixing two solutions of Na_2HPO_4 and KH_2PO_4 0.067 M (3:2 v/v) so that the pH was ~7. The wavelength used was 280 nm according to compound UV spectrum. The analyses were carried out at 25 °C.

GC-ECD determination of Dichlofluanid, Iprodione, Procymidone and Vinclozolin: The final extracts were filtered through a 0.45 μm pore size PTFE membrane and analysed by capillary gas chromatography with electron-capture detection (GC-ECD). The capillary column used was a SPB-5 ($30\,m \times 0.25$ mm i.d., 0.25 μm film thickness). The carrier gas (hydrogen) flow rate was kept constant during the run (1 ml/min, measured at 100 °C). The oven temperature program was as follows: initial temperature 100 °C, held for 1 min; 100–180 °C rate 10 °C/min; 180–320 °C rate 3 °C/min to 320 °C. The temperature of the injection port was 250 °C, while that of the detector was 300 °C. Fungicide detection was done by comparing retention times of compound standard solutions prepared at concentrations between 0.2 and 4 mg/l in methanol. Fungicide quantification was done by interpolation from the linear calibration graphs obtained in that range.

Chromatographic determination of fungicide residues was done in white and red bottled-wine samples. In order to determine the active compound transfer rate from grapes to wine, fungicides (trademark compounds) were applied 3 weeks before vintage (period recommended) and followed during the fermentation process. The presence of fungicides was evaluated in (i) musts prepared from grapes obtained in the same day, 2 and 15 days after compounds application; (ii) musts prepared from grapes after the vintage; (iii) musts obtained during and at the end of wine fermentation.

2.3. In vitro fungicide toxicity assays

Micro-organisms: *Saccharomyces cerevisiae* ESA1 and *Rhodothorula glutinis* ESA7, isolated from wine and *Zygosaccharomyces rouxii* ESA8, isolated from honey were obtained in Microbiology Laboratory of Escola Superior Agrária de Bragança. *Candida utilis* IGC2578 was obtained from the Portuguese Yeasts Culture Collection (PYCC) of Universidade Nova de Lisboa. *Zygosaccharomyces bailii* ISA1025, isolated from wine was obtained from the Microbiology Laboratory of Instituto Superior de Agronomia de Lisboa.

Fungicides used in the present study were Benomyl, Dichlofluanid, Iprodione, Procymidone and Vinclozolin (analytical standard compounds). Due to their low solubility in water, all compounds were dissolved in DMSO at concentrations ranging from 2 to 5×10^{-4} g/l using several dilutions steps, and were sterilized by filtration.

A minimal mineral liquid medium with vitamins (Uden, 1967) with 2% of glucose (w/v) was used to prepare the inocula after being sterilized in an autoclave (121 °C, 20 min). Erlenmeyer flasks (150 ml) with 50 ml of the liquid culture medium were inoculated with the yeast suspension (10^8 cfu/ml) and each concentration of fungicide to be tested was added. Incubation was carried out for 6 days at 25 °C in a rotary shaker at 150 rpm. The growth of yeast cultures was monitored by measuring optical density at 640 nm in a spectrophotometer UV–visible. Controls were carried out in the same conditions but in the absence of fungicides and in the presence of only the solvent DMSO. All assays were carried out under aseptic conditions. Tests were performed in duplicate and X_{min} (fungicide concentration that inhibited 10% of the yeasts growth) and X_{max} (fungicide concentration that inhibited 90% of the yeasts growth) were determined by linear regression analysis and expressed as the mean of the duplicate values.

2.4. In vivo microvinification assays

Micro-organisms: *S. cerevisiae* ESA1; *S. cerevisiae* MB and MT, isolated from white and red wines respectively, were obtained by Universidade Nova de Lisboa.

Fungicides Benomyl and Dichlofluanid (trademark compounds) were applied in a concentration of 2 mg/l of must as recommended in the label.

The grapes (40 Kg) were collected to open plastic boxes and then transported to Adega Cooperativa de Murça for microvinification assays. The pressing of grapes was done at 18 °C and the mixture obtained was transferred to inox fermentation wine vats (25-l capacity). The sulphurous solution (60 mg/l), the fungicide (2 mg/l of must) and the yeast to be studied were added to each wine vat. During the alcoholic fermentation the mixture was re-pressed three times by day and the temperature and density values were measured. At the end of the process the wine was racked to glass demijohn (10-l capacity) for the malolactic fermentation. The wine was racked again to glass demijohn (5-l capacity) and the pH was corrected with tartaric acid. After clarification and before bottling, several analyses were performed. Physical and chemical analyses were done according to the methods recommended by the Organization Internationale de la Vigne et du Vin; density, temperature, pH, alcoholic grade, volatile acidity, total acidity, total SO_2 and free SO_2 values were determined. The organoleptic analysis was done by a group of five tasters and three parameters were evaluated in different scales: color, 1–3; aroma; 1–4; taste, 1–6 and global impression, 1–7.

3. Results and discussion

3.1. Chromatographic determination of fungicide residues in grape must or wine samples

Several red and white bottled wines from the regions of Trás-os-Montes and Entre Douro e Minho were analysed for some of the most common fungicides. From the fungicides detected, Benomyl was the most frequently found while Dichlofluanid was not detected in our analyses. The percentage of the samples in which the presence of fungicides was detectable and the levels detected are presented in Table 1 (red bottled-wines) and Table 2 (white bottled-wines). The bottled wines from Entre Douro e Minho region more frequently had detectable levels of the fungicides.

Table 1
Fungicide residues detected in red wine-bottled samples

Results	Producing region			
	Trás-os-Montes		Entre Douro e Minho	
	Analyzed samples (no.) 50		Analyzed samples (no.) 15	
	a (%)	*b* (mg/l)	*a* (%)	*b* (mg/l)
Fungicide	14	0.0175	100	3.46
Benomyl	18	0.019	33	0.030
Iprodione	10	0.014	67	0.072
Procymidone	0	<0.0005	0	<0.0005
Dichlofluanid	14	0.015	100	0.011
Vinclozolin				

(a) Percentage of samples in which was detected the presence of fungicide residues.
(b) Fungicide quantitative levels (mg/l) detected. The results are expressed as the mean of the analyzed samples.

Table 2
Fungicide residues detected in white wine-bottled samples

Results	Producing region			
	Trás-os-Montes		Entre Douro e Minho	
	Analyzed samples (no.) 15		Analyzed samples (no.) 51	
	a (%)	*b* (mg/l)	*a* (%)	*b* (mg/l)
Fungicide	27	0.127	100	1.23
Benomyl	20	0.107	57	0.068
Iprodione	27	0.147	39	0.0376
Procymidone	0	<0.0005	0	<0.0005
Dichlofluanid	13	0.147	65	0.098
Vinclozolin				

(a) Percentage of samples in which was detected the presence of fungicide residues.
(b) Fungicide quantitative levels (mg/l) detected. The results are expressed as the mean of the analyzed samples.

The active compound transfer percentages from grapes to wine are presented in Fig. 1. Benomyl concentration was almost the same in all the process phases. This is consistent with the observation that this compound was the most frequently detected in wine bottled samples (100% in wines from Entre Douro e Minho region) (Tables 1 and 2). Dichlofluanid was not detectable after the initial phases of the process. Again this is consistent with the observation that this compound was not also detected in any of the analysed bottled wines (Tables 1 and 2). The percentages of active compound in wine after the fermentation process were 20% and 30% for Procymidone and Iprodione, respectively.

3.2. Evaluation of in vitro fungicide toxicity effects

The growth of several wine-fermentation yeasts, *S. cerevisiae*, *R. glutinis*, *Zygosaccharomyces bailli*, *Z. rouxii* and *C. utilis* was evaluated in presence of DMSO fungicide solutions (Iprodione, Procymidone, Dichlofluanid and Benomyl) at different concentrations. DMSO was chosen as solvent after comparative toxicity assays (data not shown) and was not toxic. The results obtained are presented in Table 3.

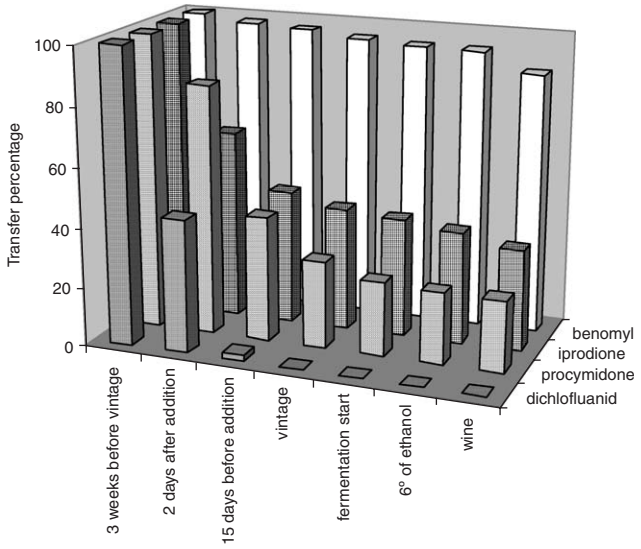

Fig. 1. Active compound transfer percentage along fermentation process. 100% is correspondent to 1.60 mg/l for benomyl, 1.69 mg/l for iprodione, 1.50 mg/l for procymidone, 1.98 mg/l for dichlofluanid.

The presence of fungicides in the culture medium at concentrations above X_{min} induced the inhibition of yeasts growth. This effect was increased for higher fungicide concentrations. According to the fungicide concentration that inhibited 90% of the yeasts growth (X_{max}), *R. glutinis* was the most susceptible to negative effects of the tested compounds, while *Z. rouxii* and *S. cerevisiae* were more resistant. Dichlofluanid and Benomyl were the most toxic fungicides.

3.3. Evaluation of in vivo fungicide toxicity effects

To evaluate the effect of fungicides in the (i) time spent in fermentation start and terminus; (ii) physical and chemical parameters of wine and (iii) organoleptic characteristics of wine, several microvinifications conducted by different *S. cerevisiae* stirpes were performed in the presence and absence of Benomyl and Dichlofluanid. Benomyl was selected because it was most frequently detected by us in bottled wines remaining for a longer time along the process, and presented the higher transfer percentage. Dichlofluanid was chosen because it was the most toxic for the yeasts (see above). The results obtained for the physical and chemical parameters, for the global organoleptic analysis and for the fermentation start and terminus times are presented in Tables 4 and 5.

Only Benomyl showed a slight delay in the initiation of fermentation, possibly probably due to its toxicity. The physical and chemical parameters did not change with the presence of fungicides. The organoleptic characteristics were also not affected by the presence of those compounds but revealed a dependence on the yeast species used.

On the basis of the above findings, it is concluded that the presence of fungicide active substances showed in vitro negative effects over several yeasts (*Saccharomyces* and non-*Saccharomyces*) usually present in the fermentation process, while in the microvinification assays only

Table 3
Fungicide concentration that inhibited 10% of the yeasts growth (X_{min}) and Fungicide concentration that inhibited 90% of the yeasts growth ($X_{máx}$)

Yeast	Fungicide solution in DMSO (g/l)							
	Procymidone		Benomyl		Dichlofluanid		Iprodione	
	X_{min}	$X_{máx}$	X_{min}	$X_{máx}$	X_{min}	$X_{máx}$	X_{min}	$X_{máx}$
S. cerevisiae	0.15	2.00	0.10	0.75	0.0005	0.0025	0.10	1.60
R. glutinis	0.10	0.80	0.05	0.75	0.0005	0.002	0.10	1.25
Z. bailli	0.15	0.80	0.05	0.75	0.0005	0.002	0.10	1.50
C. utilis	0.15	1.60	0.02	0.25	0.0005	0.0025	0.05	1.00
Z. rouxii	0.15	2.50	0.10	0.50	0.0005	0.0025	0.10	1.60

Table 4
Physical, chemical and organoleptic parameters of red wines in the absence or in the presence of Dichlofluanid and Benomyl

Fungic.	Yeast	Density	Grade (°)	pH	Total acidity ($g_{H_2SO_4}/l$)	Volatile acidity (g_{CH_3COOH}/l)	Free SO_2 (mg/l)	Total SO_2 (mg/l)	Ferm. start (days)	Ferm. end (days)	T_{max} (°C)	Org. leptic test
0 mg/l	Y1	994.3	12.1	3.49	6.20	0.36	18	69	4	20	21	10.1
	Y2	993.8	12.3	3.53	6.40	0.45	19	60	3	13	20	11.8
	Y3	993.8	12.2	3.54	6.30	0.48	21	64	5	20	21	12.8
	Y4	993.8	12.2	3.49	6.50	0.48	17	59	5	20	20	10.7
Dichlof. (2 mg/l)	Y1	993.5	12.1	3.50	6.10	0.37	18	68	4	20	20	10.0
	Y2	994.0	12.2	3.54	6.40	0.46	19	60	4	14	20	11.7
	Y3	993.7	12.4	3.51	6.40	0.48	21	64	5	20	21	12.8
	Y4	993.8	12.2	3.49	6.50	0.49	18	59	5	20	20	11.0
Benom. (2 mg/l)	Y1	994.2	12.0	3.52	6.30	0.37	18	70	6	20	21	9.8
	Y2	994.1	12.3	3.51	6.40	0.47	19	63	5	16	21	11.2
	Y3	993.7	12.1	3.50	6.40	0.49	22	65	6	21	21	13.3
	Y4	992.9	12.0	3.50	6.49	0.47	17	59	6	20	20	11.0

Y1—Control (Trademark yeast); Y2—*Saccharomyces cerevisiae* ESA1; Y3—*Saccharomyces cerevisiae* MT; Y4—*Saccharomyces cerevisiae* MB.

Table 5
Physical, chemical and organoleptic parameters of white wines in the absence or in the presence of Dichlofluanid and Benomyl

Fungic.	Yeast	Density	Grade (°)	pH	Total acidity ($g_{H_2SO_4}/l$)	Volatile acidity (g_{CH_3COOH}/l)	Free SO_2 (mg/l)	Total SO_2 (mg/l)	Ferm. start (days)	Ferm. end (days)	T_{max} (°C)	Org. leptic test
0 mg/l	Y1	990.6	10.2	3.20	6.44	0.39	19.0	109	4	15	19	12.4
	Y2	990.4	10.3	3.22	6.52	0.48	14.0	103	3	15	20	10.5
	Y3	990.4	10.1	3.23	6.34	0.42	13.0	115	3	14	20	14.9
	Y4	990.3	10.3	3.19	6.72	0.39	7.0	106	3	14	20	11.8
Dichlof. (2 mg/l)	Y1	990.7	10.1	3.23	6.39	0.40	18.0	110	4	16	20	11.9
	Y2	990.5	10.2	3.22	6.50	0.45	13.6	105	3	15	20	9.9
	Y3	990.4	10.0	3.23	6.33	0.41	13.0	116	3	15	20	14.6
	Y4	990.3	10.3	3.20	6.73	0.39	8.0	107	3	14	20	11.2
Benom.(2 mg/l)	Y1	990.8	9.9	3.20	6.40	0.40	18.0	109	6	16	19	12.0
	Y2	990.6	10.0	3.20	6.54	0.49	15.0	103	5	15	20	10.3
	Y3	990.5	9.8	3.23	6.40	0.46	13.0	117	5	14	20	14.8
	Y4	990.4	10.1	3.20	6.70	0.40	8.0	108	5	15	20	11.6

Y1—Control (Trademark yeast); Y2—*Saccharomyces cerevisiae* ESA1; Y3—*Saccharomyces cerevisiae* MT; Y4—*Saccharomyces cerevisiae* MB.

Benomyl showed a delay effect in the fermentation start. The presence of fungicides itself apparently had no influence in the wine characteristics. However, as the levels of fungicides detected in the wine-bottled samples were higher than the admissible (Benomyl and Dichlofluanid- 0 mg/l; Iprodione- 0.01 mg/l; Procymidone- 0.005 mg/l), we recommend the rigorous application of fungicides, both in terms of the recommended dose or the recommended application period.

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