

## Effects of Clarification and Filtration Processes on the Removal of Fungicide Residues in Red Wines (Var. Monastrell)

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The effects of six clarification agents (egg albumin, blood albumin, bentonite plus gelatin, charcoal, PVPP, and silica gel) on the removal of residues of four fungicides (cyprodinil, fludioxonil, pyrimethanil, and quinoxyfen) applied directly to a racked red wine, elaborated from Monastrell variety grapes from the D.O. region of Jumilla (Murcia, Spain), are studied. The clarified wines were filtered with 0.45  $\mu\text{m}$  nylon filters to determine the influence of this winemaking process in the disappearance of fungicide residues. Analytical determination of cyprodinil, fludioxonil and pyrimethanil was performed by gas chromatography with an alkaline thermoionic detector (NPD), whereas that of quinoxyfen using an electron captor detector (ECD). In general, and for all of the fungicides except quinoxyfen, blood albumin has proved to be the most effective clarifying agent in the removal of residues, whereas silica gel proved to be ineffective against all of the pesticides with the exception of fludioxonil. Quinoxyfen is the least persistent fungicide in the clarified wines and that which appears with highest frequency in the lees. In general, filtration is not an effective step in the elimination of wine residues. The greatest elimination after filtration is obtained in wines clarified with charcoal and the lowest in those clarified with PVPP.

**KEYWORDS:** Fungicide residues; clarifying substances; filtration; red wine

### INTRODUCTION

For a long time the scientific and technical progress in the wine industry has been centered mostly on efforts to stabilize wine and thus avoid the appearance of precipitates, as this is viewed negatively by the consumer. Indeed, well-known precipitations account for a large number of wines returned by customers.

In general, the existence of deposits of crystals and/or colorant matter in the wines is not acceptable regardless of the age of the wine; the demand is for clarity. Hence, the clarification and stabilization of wines are performed to avoid any defects in clarity or any physical–chemical or microbiological disequilibrium (1, 2).

Clarification may be natural (progressive deposit with formation of lees), although the winemaker usually accelerates this natural phenomenon to obtain the degree of clarity desired. Likewise, stabilization is aimed at conserving the clarity and at preventing deviations or accidents during the preservation of the wine, without stunting its normal development. The two processes are complementary and are performed prior to bottling (3–5).

Clarification is usually facilitated by the use of mineral products, such as bentonite, organic products, such as gelatin and egg and blood albumins, and also synthetic materials, such as polyvinylpyrrolidone (PVPP), a product specifically

developed to this end (6–12). Silicate suspensions and enological tannins also take part in the clarification, although they are not considered as clarifying agents in their own right but as aids to the process, especially when performed with mineral or organic products (13). Other substances, such as charcoal, have reduced clarifying power but do correct color, thus improving the appearance of the product (14).

Filtration is likewise considered to be a stabilization operation because it eliminates those substances and microbial agents that after clarification may, respectively, dirty or alter the wine. The most common method of performing filtration is through the use of nylon filters or other materials of a specific pore size.

All of these treatments are of great interest, not only because of the clarity demanded of wines but also because they lead to the removal (along with either the materials removed or the clarifying agents) of other exogenous substances, such as pesticide residues (15–25). The presence of these in the wine is due to the phytosanitary treatments applied during the growth cycle of the vine to control diseases and plagues that would otherwise reduce both the quantity and quality of the harvests.

Given all of the above, this work studies the effects of the aforementioned agents and those of filtration using 0.45  $\mu\text{m}$  nylon filters on the removal of residues of four commonly used fungicides (cyprodinil, fludioxonil, pyrimethanil, and quinoxyfen) in the control of cryptogamic vineyard diseases in order to improve the hygienic and sanitary characteristics of the wines. We seek to contribute new information to this area of study,

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**Table 1.** Enological Parameters Analyzed in Wine<sup>a</sup>

density	pH	total acidity	volatile acidity	SO <sub>2</sub> total	SO <sub>2</sub> free	color	tone	alcoholic grade
992.30	3.50	7.71	0.16	61.0	38.0	15.18	0.57	13.0

<sup>a</sup> Density, g/L; total acidity, g/L tartaric acid; volatile acidity, g/L acetic acid; SO<sub>2</sub> total and free, mg/L; alcoholic grade, % v/v ethanol; color, (Abs420 + Abs520 + Abs620); tone, (Abs420/Abs520).

**Table 2.** Clarifying Substances, Mercantile (Commercial) Subscription, and Treatment Dose

clarifying substance	subscription	commercial name	dose
egg albumin <sup>a</sup>			
blood albumin	agrovin	Cristadolcine E.F	10 g/hL
bentonit	agrovin	Bentonita Bengel	50 g/hL
gelatin	agrovin	Stargel	6 mL/hL
charcoal	agrovin	Croer D-230	50 g/hL
PVPP	agrovin	Divegan-W	15 g/hL
silica gel	agrovin	Silisol	70 mL/hL

<sup>a</sup> Fresh egg was used for clarification. The equivalent dose is one beaten egg white per hectoliter of wine.

**Table 3.** Fungicides Studied and Treatment Doses

active ingredient	formulation	dose	water solubility, mg/L (25°C)	MRL, <sup>a</sup> mg/kg
cyprodinil	Switch WG	100 g/hL	20	2
fludioxonil	Switch WG	100 g/hL	1.8	1
pyrimethanil	Scala 40% SC	200 cm <sup>3</sup> /hL	121	5
quinoxifen	Arius 25% SC	30 cm <sup>3</sup> /hL	0.116	1

<sup>a</sup> Established for wine grapes by Spanish legislation (RD 280/1994, BOE 03/09/94).

which does not abound in the literature, because we consider it important for consumers and producers alike.

## MATERIALS AND METHODS

**Wine.** A racked red wine elaborated from Monastrell variety grapes was used. The main enological parameters analyzed are shown in **Table 1**.

**Clarifying Agents.** Commercially prepared blood albumin, bentonite, gelatin, charcoal, PVPP, and silica gel purchased from Agrovin (Ciudad Real, Spain) were used. Fresh eggs were used in the clarification with egg albumin. The doses applied are given in **Table 2**. Nylon filters (0.45 μm pore size) were used for filtration (Supelco Inc., Bellefonte, PA).

**Fungicides and Reagents.** Cyprodinil [*N*-(4-cyclopropyl-6-methylpyrimidin-2-yl)aniline], fludioxonil [4-(2,2-difluoro-1,3-benzodioxol-4-yl)pyrrole-3-carbonitrile], pyrimethanil [*N*-(4,6-dimethylpyrimidin-2-yl)aniline], and quinoxifen (5,7-dichloro-4-quinolyl-4-fluorophenyl ether) analytical standards were purchased from Novartis Agro (cyprodinil and fludioxonil), Dr. Ehrenstorfer (pyrimethanil), and Dow Agro Sciences (quinoxifen). These chemicals were at least 98.5% pure. Acetone, dichloromethane, hexane, isooctane, and toluene used were for pesticide residues (SDS), and sodium chloride and ethanol were used for analytical grade (Panreac).

**Fungicide Treatments, Clarification, and Sampling.** Before the fungicides were applied, the wine was put into 2-L containers in measures of 1.5 L. At the same time a hydroalcoholic solution was prepared from the active materials from the four fungicides at concentrations (0.5, 1, 1.75, and 1.75 mg/kg for quinoxifen, cyprodinil, fludioxonil, and pyrimethanil, respectively) that were calculated according to the commercially recommended doses for each formula (**Table 3**). These were then added through intense stirring to each of the containers. Two hours later, the corresponding clarifying agent was

**Table 4.** Amount (Milligrams) of Fungicide and Percentage Remaining (*N* = 3) in the Whole Weight of Sample (Kilograms) for Each Control Stage in the Clarification Process with Egg Albumin

stage	clarification with egg albumin							
	cyprodinil		fludioxonil		pyrimethanil		quinoxifen	
	mg	%	mg	%	mg	%	mg	%
wine	1.650	100	2.616	100	2.603	100	0.791	100
clarified wine	1.200	72.73	2.354	89.98	1.767	67.88	0.389	49.18
lees	0.051	3.09	0.042	1.61	0.034	1.31	0.090	11.38
filtered wine	1.112	67.39	1.329	50.80	1.650	63.39	0.389	49.18

added to each container. The first assay was with fresh egg albumin (EA), prepared from beaten egg white. The second was with powdered blood albumin (BA). The third was with bentonite, which had been previously expanded with water and liquid gelatin at 28% (BG). The fourth was with charcoal (C). The fifth was with PVPP (P), and the sixth was with silica gel (S).

All of the clarifying agents were added with intense stirring for some minutes. The containers were then sealed and left to settle for 5 days. Once the clean wines had been racked, they were filtered through nylon 0.45 μm pore filters. All assays were performed three times.

Samples of the nonclarified and clarified wine were taken from the lees and from the filtered wine for fungicide residue analysis.

**Extraction Procedure, Chromatographic Analysis, and Method Validation.** Extraction, identification, and quantification of cyprodinil, fludioxonil, pyrimethanil, and quinoxifen were performed according to the methods described by Navarro et al. (26) and Fernández et al. (27).

## RESULTS AND DISCUSSION

Tables 4–9 show the average quantities of fungicide (milligrams) in the weight or total volume of the sample and the remaining percentages corresponding to each step of the clarification and filtration process. These quantities were calculated from the concentrations detected in each sample and according to the results of each stage (liters of wine clarified and filtered and kilograms of lees). Remaining percentages were calculated by taking as 100% the total amount of residues present in the nonclarified wine.

The fresh egg albumin is the clarifying agent used most in high-prestige red wines. It removes a large number of phenols and mellows the rich wines in astringent tannins. Compared to other clarifying agents, it offers the advantage of not modifying the sensory qualities. However, it is not as widely used as other clarifying agents (4, 5). As **Table 4** shows, clarification with fresh egg albumin leads to the elimination along with the lees of 11.38% of the quinoxifen residues and only 1.31% of those of pyrimethanil. Fludioxonil and cyprodinil are found in higher proportions in clarified wines than in the lees (89.98 versus 1.61% and 72.73 versus 3.09%, respectively). Quinoxifen is the fungicide that is removed most with this clarifying agent (49.18% of the remaining residues).

The use of blood albumin is recommended for young red highly tannic wines, because it is a powerful clarifying agent. Compared to other protein-clarifying agents used in wine processes, it has shown optimal behavior in all types of clarifications (5, 9). Thus, better results are obtained with blood albumin (**Table 5**) for pyrimethanil and cyprodinil, with 31.27 and 32.02% of the respective residues being found in wines clarified with this agent. Nevertheless, the clean wine retains ~71% of fludioxonil traces and ~60.50% in the case of quinoxifen. In contrast to clarification with egg albumin, quinoxifen is one of the fungicides that presents the greatest residues in the wines.

**Table 5.** Amount (Milligrams) of Fungicide and Percentage Remaining ( $N = 3$ ) in the Whole Weight of Sample (Kilograms) for Each Control Stage in the Clarification Process with Blood Albumin

stage	clarification with blood albumin							
	cyprodinil		fludioxonil		pyrimethanil		quinoxifen	
	mg	%	mg	%	mg	%	mg	%
wine	1.599	100	2.489	100	2.588	100	0.728	100
clarified wine	0.512	32.02	1.767	70.99	0.809	31.27	0.440	60.44
lees	0.046	2.89	0.046	1.86	0.030	1.15	0.055	7.55
filtered wine	0.511	31.95	1.616	64.93	0.746	28.83	0.381	52.34

**Table 6.** Amount (Milligrams) of Fungicide and Percentage Remaining ( $N = 3$ ) in the Whole Weight of Sample (Kilograms) for Each Control Stage in the Clarification Process with Bentonite Plus Gelatin

stage	clarification with bentonite plus gelatine							
	cyprodinil		fludioxonil		pyrimethanil		quinoxifen	
	mg	%	mg	%	mg	%	mg	%
wine	1.606	100	2.458	100	2.514	100	0.788	100
clarified wine	0.973	60.60	2.342	95.27	1.576	62.72	0.315	39.97
lees	0.067	4.20	0.049	1.99	0.044	1.75	0.096	12.18
filtered wine	0.763	47.51	1.551	63.10	1.583	62.96	0.299	37.94

Bentonite is a natural montmorillonite clay consisting of aluminum and silicon oxides in small flat plates, which separate when rehydrated, thus creating a large absorptive surface area (2, 8). This surface area is negatively charged, allowing for ion exchange and other electrostatic interactions, whereas hydrogen bonding is possible at the edges of the plates. Its main use in winemaking is the elimination of positively charged proteins, because this is, today, the only efficient system to prevent protein breakdown, and hence its stabilizing effect through the removal of unstable proteins from the wine is more important than the clarification it produces (4). It is, therefore, used with gelatin to enhance the clarifying action (2, 6, 28). Gelatin should never be used alone because it leads to a slight enrichment of proteins, especially in low tannin content wines (white wines) (5).

However, the use of bentonite may alter the sensory quality of the wine through various processes: reduction in the concentration of peptide-associated polyphenols (29); reaction with cationic anthocyanins, which are responsible for the hue in red wines (2); reduction of color intensity (1, 5, 8); adsorption of aroma components (1, 8, 30); etc.

Our study shows that the combination of bentonite and gelatin is not effective in eliminating fludioxonil residues (**Table 6**), with very high percentages of this fungicide remaining in the clarified wines (95.27%). Cyprodinil and pyrimethanil are found in similar percentages (~60–63%), whereas only 39.97% of quinoxifen residues appear.

The clarifying effect of charcoal is very weak. It does not produce flocculation of the colloids, which cause the cloudiness. Its use is directed toward decoloring and deodorizing wines, although it has a high affinity for benzenoid and nonpolar substances. It is also used to remove the browning components (2). In our experiment, clarification with charcoal (**Table 7**) serves to remove the majority of quinoxifen residues, because the remnants in the clarified wine stand at 18.45%. It is not very effective for the other fungicides; 80.43 and 90.38% remnants for cyprodinil and fludioxonil, respectively, remain. The residual proportions in lees for this product are clearly in excess of those found in the other clarifications, ranging from 9.66 to 15.28% (pyrimethanil and cyprodinil, respectively).

**Table 7.** Amount (Milligrams) of Fungicide and Percentage Remaining ( $N = 3$ ) in the Whole Weight of Sample (Kilograms) for Each Control Stage in the Clarification Process with Charcoal

stage	clarification with charcoal							
	cyprodinil		fludioxonil		pyrimethanil		quinoxifen	
	mg	%	mg	%	mg	%	mg	%
wine	1.611	100	2.707	100	2.491	100	0.791	100
clarified wine	1.295	80.43	2.446	90.38	2.065	82.90	0.146	18.45
lees	0.246	15.28	0.382	14.14	0.240	9.66	0.094	11.88
filtered wine	0.427	26.51	nd <sup>a</sup>		0.892	35.81	0.141	17.83

<sup>a</sup> Not detectable.

**Table 8.** Amount (Milligrams) of Fungicide and Percentage Remaining ( $N = 3$ ) in the Whole Weight of Sample (Kilograms) for Each Control Stage in the Clarification Process with PVPP

stage	clarification with PVPP							
	cyprodinil		fludioxonil		pyrimethanil		quinoxifen	
	mg	%	mg	%	mg	%	mg	%
wine	1.559	100	2.569	100	2.680	100	0.726	100
clarified wine	1.420	91.09	1.786	69.53	2.261	84.37	0.400	55.11
lees	0.112	7.16	0.174	6.78	0.069	2.59	0.177	24.44
filtered wine	1.285	80.40	1.787	69.57	2.265	84.52	0.403	55.59

**Table 9.** Amount (Milligrams) of Fungicide and Percentage Remaining ( $N = 3$ ) in the Whole Weight of Sample (Kilograms) for Each Control Stage in the Clarification Process with Silica Gel

stage	clarification with silica gel							
	cyprodinil		fludioxonil		pyrimethanil		quinoxifen	
	mg	%	mg	%	mg	%	mg	%
wine	1.590	100	2.570	100	2.523	100	0.809	100
clarified wine	1.584	99.66	2.137	83.15	2.325	92.14	0.491	60.69
lees	0.026	1.61	0.030	1.17	0.015	0.61	0.068	8.41
filtered wine	1.344	84.53	1.898	73.86	2.307	91.44	0.375	46.35

PVPP is a synthetic, high molecular weight, cross-linked polymer of poly(vinylpyrrolidone), and its mechanism of absorption is through hydrogen bonding between the carbonyl groups of the polyamide and phenolic hydrogens (1, 10). The interest in PVPP for winemaking lies in the specific nature of its action on the polyphenols in wine, to the extent that due to its insolubility in hydroalcoholic media its application is aimed at the removal of those phenol compounds which produce, through condensation, oxidative alterations in wine (catechins and other flavonoids). Thus, it affects both taste and hue, lessening the sharpness and browning in white wines and highlighting the hue of red wines of excessive tannin content (5, 31).

Finally, silica gel is more an assistant clarifying agent than one in its own right. It is adapted to enhance the clarification of other agents, such as gelatin. Among its properties we emphasize its high capacity to fix tannin substances and polyphenols that cannot be removed by other clarifying agents (10).

The addition of PVPP and silica gel (**Tables 8 and 9**) produced clarified wines with the highest concentrations of remnants of cyprodinil (91.09 and 99.66%, respectively) and the lowest for quinoxifen (55.11 and 60.69%, respectively). Although residue removal is low in both processes, it is better in that performed with PVPP.

From the data in **Tables 4–9** it is deduced that in all of the assays, except that with blood albumin, quinoxifen is the least

persistent fungicide in the wines and, in turn, that which is most detected in the lees. Only in those coming from the clarification with charcoal have slightly higher remnants of cyprodinil and fludioxonil than those of quinoxifen been obtained (15.28, 14.14, and 11.88%, respectively). A relationship therefore seems to exist between the percentage removed and the fungicide's solubility in water—the lower the solubility and polarity, the greater the removal. Ruediger et al. (23) came to the same conclusion in a study on 10 pesticides (7 fungicides and 3 insecticides), different from those studied here. However, fludioxonil, which is the second least soluble product in water (32), does not fit in with this norm, which implies that its removal depends, in general, on the nature of the product, although the product's solubility and polarity may be highly influential within this condition.

The order of effectiveness of the different clarifying agents on the four fungicides was as follows:

#### cyprodinil

blood albumin > bentonite + gelatin > egg albumin > charcoal > PVPP > silica gel

#### fludioxonil

PVPP > blood albumin > silica gel > egg albumin > charcoal > bentonite + gelatin

#### pyrimethanil

blood albumin > bentonite + gelatin > egg albumin > charcoal > PVPP > silica gel

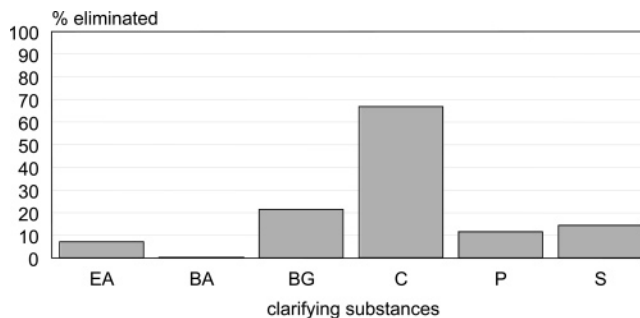
#### quinoxifen

charcoal > bentonite + gelatin > egg albumin > PVPP > blood albumin > silica gel

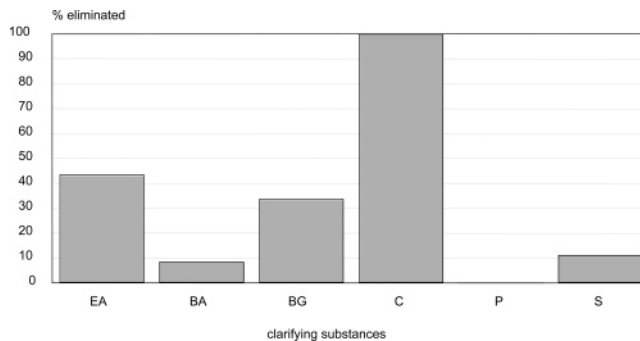
Overall, the most effective clarifying agent is blood albumin. Nevertheless, it should be taken into account that we are dealing with a very energetic agent, the use of which has important drawbacks, such as undesired tastes and soluble proteins that may lead to cloudiness. On the other hand, with the exception of fludioxonil, clarification with silica gel proves to be insufficient to reduce significantly the residual contents of fungicides in clarified wines.

According to the authors, the best option is the use of bentonite and gelatin, because they considerably reduce the cyprodinil, pyrimethanil, and quinoxifen contents and do not generate the drawbacks described above. Furthermore, the action of both products depends on the pH of wine, with an optimum for gelatin of between 3.2 and 3.7 units, as is the case here (pH 3.5). At this pH, cyprodinil and pyrimethanil, which have  $pK_a$  values of 4.44 and 3.52, respectively (32), are positively charged and therefore have a greater potential to bond with bentonite through electrostatic forces. Fludioxonil, however, with a  $pK_a$  of <0 (32), is mainly found in wine in its neutral form and cannot bond with bentonite, and it presents a very low removal percentage. For the removal of fludioxonil we must use charcoal, followed by filtration for a total removal of the pesticide.

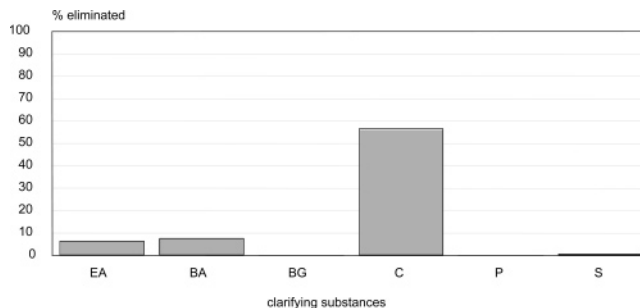
The orders of effectiveness are the same for pyrimethanil and cyprodinil. However, the remnants of pyrimethanil in lees are lower than those found for cyprodinil. This may be due to both fungicides' belonging to the same chemical family (anilino-pyrimidines), with their structures differing in just one radical. With regard to the levels of residues found in the lees, it should be noted that pyrimethanil appears in a lower proportion than



**Figure 1.** Percentage of cyprodinil residues eliminated during filtration of the wine [egg albumin (EA); blood albumin (BA); bentonite + gelatin (BG); charcoal (C); PVPP (P); silica gel (S)].



**Figure 2.** Percentage of fludioxonil residues eliminated during filtration of the wine [egg albumin (EA); blood albumin (BA); bentonite + gelatin (BG); charcoal (C); PVPP (P); silica gel (S)].



**Figure 3.** Percentage of pyrimethanil residues eliminated during filtration of the wine [egg albumin (EA); blood albumin (BA); bentonite + gelatin (BG); charcoal (C); PVPP (P); silica gel (S)].

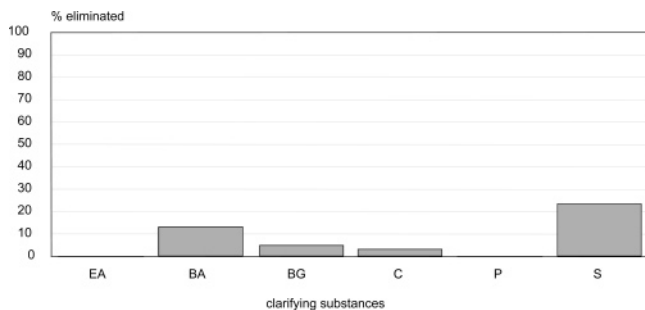
cyprodinil because of its higher solubility in hydroalcoholic mediums (32).

Another study on the clarification in wines treated with cyprodinil, fludioxonil, and pyrimethanil reveals residual reductions for cyprodinil of 42% with bentonite and 100% with charcoal. Gelatin and PVPP are ineffective against this fungicide. Charcoal also gives a 100% removal of fludioxonil residues and 92% removal of pyrimethanil residues, whereas PVPP reduces the concentration of the last pesticide by only 11%. The other products reveal no effect on the concentrations of fludioxonil and pyrimethanil (17).

**Figures 1–4** show the removal percentages for each of the active ingredients during filtration, with respect to the amount present in the clarified wines. The small differences found in the percentages of remnants indicate that filtration is not, in general, an effective step in removing residues from wine.

The highest removal percentages are produced after clarification with charcoal, which may be due to the fact that the clarifying effect is very weak and flocculation of the colloids, where fungicide residues may be retained, does not occur. After





**Figure 4.** Percentage of quinoxifen residues eliminated during filtration of the wine [egg albumin (EA); blood albumin (BA); bentonite + gelatin (BG); charcoal (C); PVPP (P); silica gel (S)].

filtering of the wine, these colloids are removed along with the residues associated with them.

The lowest removal percentages are obtained for wines previously clarified with PVPP, because this is the most insoluble product in hydroalcoholic solutions, and it is therefore practically eliminated during the clarification, with the corresponding removal of the associated pesticides.

In general, we can state that filtration of wines is much more effective for cyprodinil and fludioxonil than for the other fungicides studied.

Although we have not found references to the effects of filtration on the removal of the fungicides dealt with here in the literature, some authors have published results for other pesticides, such as metalaxyl, fenarimol, penconazole, and vinclozolin. In this vein, Navarro et al. (20) show that filtration of wines with residues of these products and which have been previously clarified with bentonite and gelatin leads to the disappearance of 2% of metalaxyl, 7% of fenarimol, 25% of penconazole, and 28% of vinclozolin. Using the same clarification treatment, rosé wines with fenarimol and penconazole filtered through nylon show a decrease of 12% in the levels of penconazole, whereas those of fenarimol remain unchanged (19).

Following the conclusions reached herein, the correct choice of clarifying agent combined with filtration is an effective way of removing a high percentage of the fungicides studied. We also consider that studies of this type are highly useful for winemakers because they ensure the hygienic and sanitary quality of wines thus elaborated while providing data for the preparation of legislation norms on maximum residues limits in wines, which might include correction factors for the winemaking processes employed.

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