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Influence of Fungicide Residues in Wine Quality.

José Oliva Ortiz, Paula Payá Peñalver and Alberto Barba Navarro.
Dept. of Agricultural Chemistry, Geology and Pedology.
University of Murcia.
Spain

1. Introduction

The cultivation of grapes may be regarded as the oldest and most relevant food sector of the world from 6000 years ago as well as the enology. During the 70s, the world's wine production was growing reaching 10.2 million Ha. Since the 80s, the planted area has decreased due to the excess of wine in the world market and the consequent eradication of vineyards has happened (-21%) reaching a total area of 7.92 million Ha in 2001 and keeping constant, though with small fluctuations in recent years (OIV, 2006). An estimated 57% of grape world production is used for winemaking, considering an average annual consumption of about 40 l of wine per person.

The vast acreage used by vineyards and climatic conditions have led to a suitable habitat for the development of large number of pests, not only responsible for the decrease in crop yield but even for the total loss itself. Control of pests and diseases of the vine is one of the pillars that must underpin profitable wine production. It should be firstly pointed out Lepidoptera *(Lobesia botrana* and *Clysia ambigüella)* and secondly the cryptogamic ones, especially *powdery* and *mildew*, and finally rot, especially the acidic and noble ones, among the major pests (insects, mites and nematodes) and diseases (fungi) affecting the cultivation of the vine (Coscollá, 1992; MAPA, 1992; Carrero, 1996).

The defense of the wine producer against this situation sometimes results in abuses which produce the presence of pesticide residues in grapes as consequence and eventually in must and wine, though the type of wine-making and its stages may influence on their disappearance. In modern agriculture, considering that organic farming is in continuous expansion, one can hardly do without the use of synthetic plant protection products to ensure a regular and substantial production of quality.

2. Effects of pesticide residues.

As a direct result of pesticide treatments carried out in viticulture, not to mention the possible environmental pollution now, we can cite three effects of their use:

- Residues in grapes, musts and wines.
- Influence on fermentation and organoleptic characteristics of wine.
- Health and hygienic quality and toxicological effect on the consumer.

2.1 Residue presence in grapes, must and wine.

The occurrence of residues of fungicides, as direct result of treatments with plant protection products during the growing season and especially between veraison and ripening of the vine, depends on such diverse factors as: products, formulation and dosage of treatment used, time between product application and harvesting, product safety time and climatological factors (sunshine, rain, etc.) (CIBA, 1993; Coscollá, 1993; Celik et al., 1995; Pimentel, 1997; Montemurro et al., 2002; Whitmyre et al., 2004).

Firstly and before entering fully into the issue of fungicide residues in wines, it is imperative to note the great development achieved by the analytical methodology in recent years which have helped to reach high sensitivities using macro and micro procedures *on- line,* solid phase micro extractions, introduction of new methods of extraction as QuEChERS, etc. (Oliva et al., 1999a; Payá et al., 2007). In terms of analytical techniques, gas chromatography (GC) and high pressure liquid chromatography (HPLC) coupled to mass detectors (MS) lead to safe and reliable results. In this field, it is also important the required implementation of quality criteria in both trials (UNE/ISO 17025 and SANCO Guidelines) and supervised experiences (GLP) for quality assurance and technical competence of laboratories undertaking pesticide residue analysis. To cite papers aimed at the development of methodologies for multi-residue determination of fungicides in grapes, must and wine by GC and selective HPLC coupled to confirmation by MS would be almost endless in this area (Navarro et al., 2000b; Oliva et al., 2000a; Agüera, et al., 2004; Anastassiades et al., 2007; Payá et al., 2007).

Experimental data available show that if pesticides are used as indicated by manufacturers, no residues higher than those set by law at the time of harvest should appear. When harvested grapes are used in wine-making, they are transported to the winery where the elaboration of wine starts by oenotechnological processes such as crushing, draining, maceration, pressing, must racking, alcoholic fermentation, racking, clarification, stabilization and filtration. In this sense, the type of wine and the correct use of oenotechnological processes can decisively influence the disappearance or elimination of pesticide residues.

An extensive review of literature shows that there are substantial losses of pesticide residues in the transition from grape to must and from this to wine. It is important to note the influence of the dissipation rate in the crop on the possible presence of residues in the wine. Moreover, oenological processes as crushing, pressing, racking, clarification and filtration are important factors in the disappearance of fungicide residues in wine. Finally, the technique of wine-making -with or without maceration-, the addition of tannins, cryomaceration, etc. also influence the disappearance or reduction of fungicide residues (Garcia & Xirau, 1994; Navarro et al., 1997; Cabras et al., 1997; Flori et al., 2000; Angioni et al., 2003; Ruediger et al., 2005; Oliva et al., 2006, 2007a & 2007b).

Thus, some studies can serve as examples in which it was confirmed that the residues of fungicides such as pyrimethanil ones in grapes were kept in must and wine but reduced to 50% for tebuconazole, though fludioxinil and kresoxim methyl residues reduce gave reductions of 50% in must and almost eliminated in the finished wine (Figure 1) (Cabras & Angioni, 2000a).

Some authors note that the crushing of grapes does not affect the disappearance of fungicides such as azoxystrobin, cyprodinil, fludioxonil, pyrimethanil and quinoxyfen (Cabras et al., 2000b; Fernández et al., 2005). After pressing, the residues of penconazole, fenarimol or vinclozolin appeared predominantly linked to the lees. However, there are fungicides such as metalaxyl that remained in a high percentage in the must due to its high solubility in water-alcohol solutions (Navarro et al., 1999).

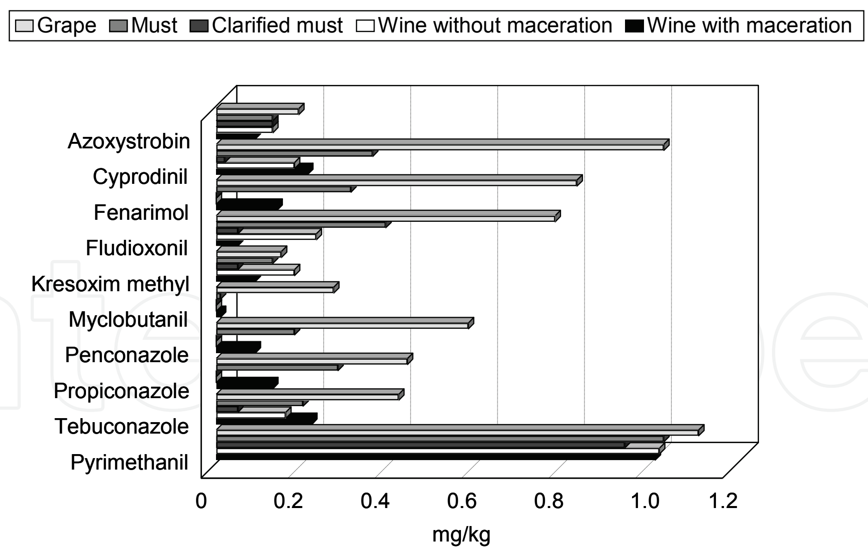

Fig. 1. Influence of the oenotechnologycal processes on the disappearance of fungicide residues

Kresoxim methyl and tetraconazole - newly marketed fungicides in viticulture- left no detectable residues in grapes after 21 days of application. Others, such as azoxystrobin, mepanipyrim and fluazinam did appear in grapes, though their residue levels decreased in must and much more in those obtained in vinifications by maceration (0.12 compared to 0.20 ppm of azoxystrobin in must with and without maceration, respectively) (Cabras et al., 1998). For treatment with quinoxyfen -very novel fungicide in powdery control, concentrations of 0.38 mg/kg were detected in grapes harvested after the application of the product (below the MRL established in Italy; 0.5 mg/kg). Fourteen days later, the residue levels had decreased to 0.09 ppm. After the vintage pressing, only 45% of the residues present in the berries were found in must. When this was subjected to centrifugation, this operation removed 8% of the level and then no residue detection was possible in the wines at the end of the fermentation process (Cabras et al., 2000b).

Moreover, the racking is a process of great impact on the disappearance of some pesticides as they can be washed and separated by lees. Also, stabilization processes such as clarification and filtration of wine can cause the disappearance of the quantities of residues present in them. The use of clarifying agents such as gelatin, bentonite, activated carbon, caseinates, etc. can drag the residues of the pesticides that due to their physical-chemical characteristics are not solved in the liquid phase. Similarly, the use of certain media such as nylon filters may significantly influence the disappearance of some pesticides (Cabras et al., 1997; Fernández et al., 2005b; Oliva et al., 2007a & 2007b).

For example, when removing the faeces suspended in must either by dynamic (centrifugation) or statically (with or without clarifying agents) 90% of sulfur and 70% of phthalimides or 40% of the dicarboximidics fungicides were lost, while water soluble products remained in the must. We must consider that the water solubility of most pesticides is low. Other products such as benzimidazols were neither eliminated in the previous process, but they disappeared when using bentonite as clarifying agent. Finally, the filtration of wine before bottling has some effect in the elimination of residues, though this is minimal (Navarro, 2000c; Soleas & Goldberg., 2000; Ruediger et al., 2004).

Comparing the evolution of residues when the wine-making is made by traditional procedures against carbonic maceration, there was greater concentration remaining in the latter process possibly because the grapes remain intact for longer time and then no transference of pesticide residues from the berries to the liquid phase occur and neither degradation reactions. However, the residual quantities after pressing were significantly less in the must from carbonic maceration.

Studying the disappearance of fungicides such as benalaxyl, metalaxyl, cyproconazole, fenarimol, penconazole, vinclozolin and mancozeb in traditional wine-making process compared to that made by carbonic maceration, it appeared that the remaining residues in the wines made by this technique were among five to ten times lower than those determined in the obtained by the traditional process. The product with the lowest rate of dissipation was metalaxyl (Navarro et al., 2000a).

In the case of traditional wine-making, remaining percentages ranged from 67 to 95% (for fenarimol and metalaxyl, respectively) during the maceration process. The first significant decline in residual levels occurred in the pressing being reduced to average values of 30% compared to the initial level in pomace, noting that the pesticide which showed higher disappearance was mancozeb. However, changes were minor in fermentation. In the lees, remaining levels were between 8-17%. Finally, clarification and filtration collaborated in the disappearance of residues (Navarro et al., 1999).

In the study of the disappearance of some fungicides on Tempranillo grape from La Rioja throughout the winemaking process, we observed the different behavior presented by the fungicides tested (Table I). Thus, procymidone and vinclozolin behaved similarly in the process of maceration and fermentation; carbendazim was not removed and dichlofluanid disappeared in about 70-80%.

Process	**Procymidone**	**Vinclozolin**	**Iprodione**	**Carbendazim**	**Dichlofluanid**
Maceration, fermentation and pressing	15%	12%	30-40%	--	70-80%
Racking	25%	45%	18-20%	30-40%	20%
Clarification	12%	13%	20%	25-30%	100%
Total	**52%**	**70%**	**70-80%**	**55-70%**	**100%**

Table I. Decrease of residues in Tempranillo grapes.

During the operations of racking and clarification, the differences were much smaller noting that dichlofluanid disappeared completely the clarification. Losses of the residues of these fungicides ranged from 50 to 100% during the winemaking process (Santos, 1997).

We can find a concrete example of the disappearance of residues during the wine production examining the study of the elimination of fenhexamid -widely used fungicide- when applied in conditions of GAP (Good Agricultural Practices) and CAP (Critical Agricultural Practices). Figures 2 and 3 show the results obtained (Barba et al., 2009b).

As we can see, the fungicide was removed by 85%during the production influencing the oenotechnological processes more than the initial concentration.

The evolution of the residue levels of four fungicides (cyprodinil, fludioxonil, pyrimethanil and quinoxyfen) during elaboration of three types of wine with maceration (traditional red wine, carbonic maceration red wine and red wine of long maceration and prefermentation at low temperature) and two types of wine without maceration (pink and white) has been studied. The disappearance curves of each fungicide have been analyzed during the period

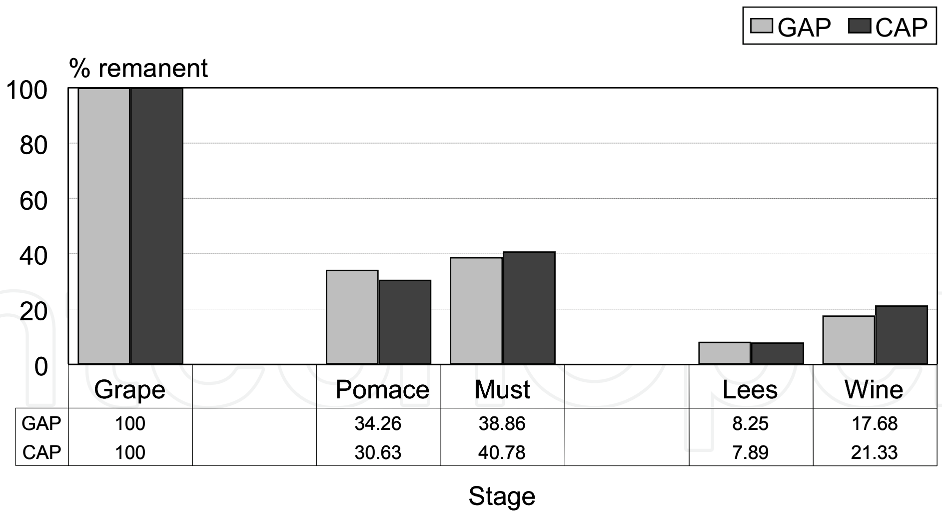

Fig. 2. Fenhexamid dissipation during the wine production.

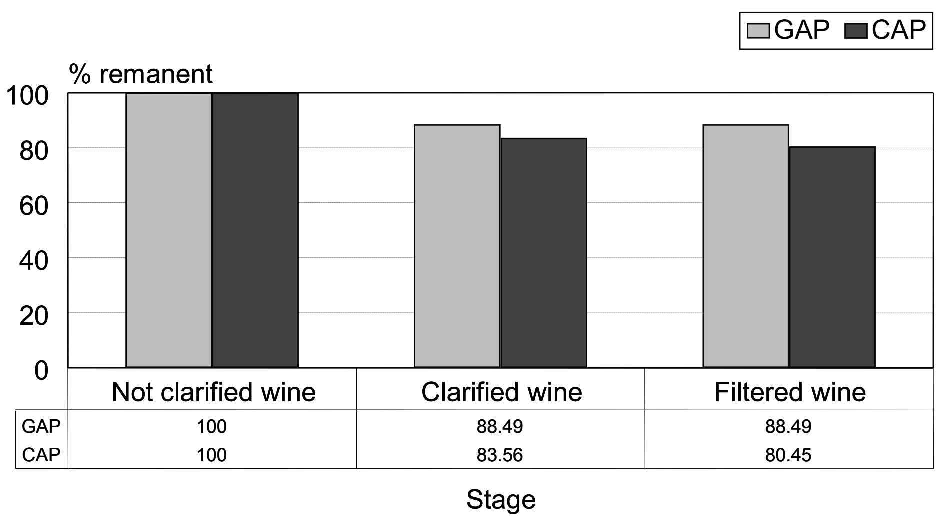

Fig. 3. Influence of clarification and filtration process in fenhexamid dissipation during the wine production.

of time of each wine-making process (21 days) and during the different steps involved in the oenological elaborations. Fludioxonil was the fastest fungicide to reduce its residual levels during the time and steps of wine-making procedures without maceration, while pyrimethanil was the lowest in practically all the cases (with and without maceration). During carbonic maceration wine-making, the decay constant of cyprodinil was greater than that of the rest pesticides in all assays (time and steps) [Fernández et al., 2005a].

The removal of recent-use fungicide (famoxadone fenhexamid, fluquinconazole, kresoxim-methyl, quinoxyfen and trifloxystrobin) can vary between 40-100% during the process of making Monastrell red wines.

Another study investigated the influence of two yeasts (*Saccharomyces cerevisiae* and *Kloeckera apiculata*) and two lactic bacteria (*Leuconostoc oenos* and *Lactobacillus plantarum*) on the degradation of six fungicides (azoxystrobin, cyprodinil, fludioxonil, mepanipyrim, pyrimethanil and tretraconazole) during alcoholic and malolactic fermentation. The results indicated that degradation occurs only during alcoholic fermentation for the fungicide pyrimethanil (20-40% decreases in the concentration of control at 10 days of fermentation.)

For the other five fungicides, the alcoholic fermentation did not produce a deletion by degradation or adsorption in yeast. Neither the bacteria studied showed degradative effect on fungicide during malolactic fermentation (Cabras et al., 1999).
There is also evidence that the copper content in the grapes and subsequent transfer to must and wine is influenced by the number of applications, the period between application and harvest and the amount of copper applied. 18% samples exceeded the MRL established (20 mg/kg) of all the wine samples analyzed in 16 Italian wineries. Therefore, it is required to set harvest time within 20 to 40-50 days. In addition, this study showed that the copper content did not depend on the strategy of pest control (conventional, integrated or organic). Of the three above factors, the most important is the amount of copper applied, since it implies about 50% of the final concentrations in grapes and wine (García-Esparza et al., 2006).
Other researchers studied the possible effect of fermentation processes on the levels of total arsenic and inorganic species [As (III) and As (V)] and organic (arsonic monomethyl acid [MMAA] and dimethyl arsinic acid [DnaA]) in 45 wines of southern Spain. The total arsenic levels were very similar for the different types of wines studied. Values in the wine samples analyzed varied between 2.1 and 14.6 mg/l. The results suggested that consumption of these wines do not have a significant contribution to the diet of total and inorganic arsenic for a moderate drinker (Herce-Pagliai et al., 2002).
Regarding the influence of storage time and temperature of wine, one study proposed the creation of an equation based on kinetic analysis that allows us to predict the concentration of ethyl-carbamate in wine after storage time at given temperature. So, you can predict the time of consumption when ethyl-carbamate levels do not exceed certain concentrations, which may lead to chronic toxicity to the consumer (Hasnip et al., 2004).
Studies on the behavior of fenhexamid in grapes during wine-making and the effect of the microflora on the alcoholic and malolactic fermentation for the degradation of fenhexamid showed that the elimination of this fungicide in the wine depends if the process is made with or without maceration. So, when there is no maceration, there is removal of 49%, while in case of maceration is 62%. Therefore, the presence of skin in contact with the must for a time produced an increase in the elimination of the fungicide studied. No effect attributed to yeast or bacteria is observed for fenhexamid degradation in wine-making (Cabras et al., 2001).
When we studied the elimination of famoxadone during wine-making, we found that there was 100% removal of the fungicide in vinification with and without maceration. Thus, levels lower than the limit of quantification (0.05 mg/kg) was achieved in wines from grapes harvested at levels close to the MRL (2 m/kg) (De Melo Abreu et al., 2006).
Removing spiroxamine is affected by the performance or not of maceration. So, when wine-making does not include maceration, removal is between 20-27%. While, removal increased to 45-62% in wines obtained with maceration. Transfer factor from grape to must was 0.55, so that half of the fungicide is removed together with the solid parts of the grape. In the case of wine to grapes, it was 0.26. In general, the elimination of residues of spiroxamine ranged 23-56%, depending on the winemaking technique used. No levels above 10% of the MRL existed in wine after treatment under GAP, so there was no toxicological risk in wines made from grapes treated with spiroxamine (Tsiropoulos et al., 2005).

2.2 Influence on the fermentation and organoleptic characteristics of wine.

Organoleptic characteristics of a wine can be considered as the result of a balance of aromatic substances and flavoring elements that compose it and that are responsible for governing the harmony between the smell and taste of wine. It is therefore extremely important to control the acid fraction (it depends on many wine properties and phenomena that take place inside), density and color (appearance, astringency and structure).

Influence on the general parameters.

To test the influence of the presence of pesticide residues on wine main features, general parameters were compared. They were: density, total acidity, volatile acidity, alcohol content and fermentation time in wines made from control grapes (with no phitosanitary treatment), traditionally cultivated grapes (typical treatments from the area of cultivation) and grapes treated with vinclozolin, fenarimol, mancozeb, metalaxyl and penconazole. In all cases, the evolution of fermentation was correct, but had no stops fermentation. The values of alcoholic grade in all wines were within the minimum values required in the DO of the area. The values and evolution of the acid fraction did not endanger its preservation or the balance of its constitution (Oliva et al., 1998a).

In another study of harvested grapes treated with the fungicide pyrimethanil, cyprodinil (Switch), fludioxonil (Switch), azoxystrobin, kresoxim methyl and quinoxyfen, vinifications were made with maceration at 5 °C for four days and six days at 25 ºC. When alcoholic fermentation was over, racking and stabilization by clarification and filtration were performed and then bottled. Determinations of the general parameters indicated in Table II were carried out in the bottled wine.

Regarding the acid fraction, it must be noted that no significant differences with respect to the control wine for the total acidity values existed in winemaking in the presence of kresoxim methyl. The same applies to the pH in the case of wine made with residues of azoxystrobin. In contrast, only wine-making in case of residues of pyrimethanil differs significantly from the control for the volatile acidity. The same happens for the alcoholic grade (Fernández et al., 2001).

	Control	Quinoxyfen (a)	Kresoxim (b)	Azoxystrobin (c)	Swicth* (d)	Pyrimethanil (e)	DS $p \leq 0.05$
TA	7.58±0.28	7.19±0.07	7.26±0.30	7.13±0.02	7.15±0.18	7.23±0.07	**a,c,d,e**
VA	0.040±0.000	0.040±0.000	0.043±0.006	0.040±0.000	0.040±0.000	0.060±0.010	**e**
pH	3.38±0.02	3.453±0.006	3.45±0.06	3.413±0.006	3.456±0.006	3.493±0.006	**a,b,d,e**
AG	12.26±0.06	12.40±0.20	12.66±0.28	12.80±0.65	12.40±0.30	13.43±0.40	**e**
CI	19.27±0.58	15.16±1.26	15.34±0.99	16.15±1.09	15.97±2.75	15.26±0.22	**a,b,c,d,e**
H	0.45±0.01	0.46±0.01	0.47±0.02	0.47±0.01	0.46±0.01	0.47±0.01	**b,c,e**
TP	2.45±0.32	1.96±0.08	1.81±0.25	2.12±0.16	1.80±0.16	1.51±0.10	**a,b,d,e**
FI	48.40±1.05	38.57±4.54	40.50±2.86	42.97±2.56	38.57±4.76	33.80±1.60	**a,b,d,e**
TPI	83.47±2.45	58.67±4.92	67.97±2.61	71.67±3.70	59.47±7.71	53.10±0.20	**a,b,c,d,e**
H_c	19.10±0.10	23.43±1.70	23.93±0.35	23.34±0.74	24.20±2.76	22.10±1.11	**a,b,c,d,e,**
S	5.26±0.02	4.87±0.54	4.17±0.10	4.33±0.13	4.27±0.50	4.66±0.32	**b,c,d,e**

TA: total acidity (g/l tartaric acid); **VA**: volatil acidity (g/l acetic acid); **pH**: pH unites; **AG**: alcoholic grade (% v/v ethanol); **CI**: Color intensity; **H**: Hue; **TP**: Total poliphenols totales (g/l galic acid); **FI**: Folín index; **TPI**: Total poliphenols index; **H_c**: Hue CIELab; **S**: Saturation; **DS**: Degree of significance; * Cyprodinil and fludioxonil mix.

Table II. Influence of some fungicides in several parameters in wine.

For all parameters responsible for the color, marked differences are seen in the wines produced in the presence of pyrimethanil. For the remaining wines, these differences are not so marked.
Considering that the higher or lower value of color intensity is strongly influenced by the degree of ripeness of the grapes, the maceration time and temperature to which it is made and if we consider that these factors have been the same in all wine-makings, it appears that the presence of the tested fungicides has influenced the diffusion of phenolic compounds from the skin to the must during maceration.
To test the influence of the fungicides myclobutanil and dichlofluanid on the production of rosé wines from Monastrell grapes, a study adding two doses (1 and 5 ppm) to the must pressed and determining later the general parameters was carried out. The results showed that although there were no stops in fermentation, the highest dose of diclofuanide did cause a delay of five days. Statistical analysis of data showed significant differences in almost all parameters, except for volatile acidity, residual density (differences in analytical significance), which indicates that all winemaking reached the end of fermentation without residual sugar and therefore not re-fermentations would not occur in the bottle, nor influenced the concentration of acetic acid in wine (Oliva et al., 1999c).
Studies performed in La Rioja (Spain) in grape Tempranillo (Rioja) treated with two doses of carbendazim, dichlofluanid, iprodione, procymidone and vinclozolin for three crops showed no significant differences in the general parameters of density, pH and total acidity. The presence of procymidone and vinclozolin decreased the concentration of malic, total polyphenols were higher in the double dose of all treatments, the red color showed higher values at double dose, but there were no differences in the shade or hue (Santos, 1997). The sensory evaluation qualifies wines as well or very well, though specific observations on development of unpleasant tastes and not certain odors were made in wines with double dose, especially in the case of carbendazim. Dichlofluanid brings smell of ethyl acetate in double doses and some appreciations in single doses.

Influence on the viability of yeast.

Among the many factors that influence the evolution of yeast flora during fermentation, the presence of pesticide residues can be highlighted by its importance as these may alter the biochemical pathways of fermentation due to its effect on synthesis reactions or inhibition of respiration or fermentation. As a result, there may be a gradual decline of the viability of population and braking of the yeast fermentation, which could lead to a complete stop of the process in severe cases (Girond et al., 1989; Frezier & Dubordieu, 1991; Doignon & Rozes, 1992; Hatzidimitrou et al., 1997; Oliva et al., 2000b; García, 2002).
It was found that sulfur has no negative action on yeast in the case of inorganic fungicides, but it may cause development of off-flavors in wine (at high concentrations). On the contrary, copper significantly inhibits the growth of *Saccharomyces cerevisiae* at concentrations of 10 ppm.
Organic products with marked fungicide character derived from sulphonamides (dichlofluanid) or phthalimides (folpet and captafol) are particularly harmful to certain strains of yeasts (*Hanseniaspora uvarum, Saccharomyces cerevisiae and Saccharomyces bayanus*) (Oliva et al., 1999c). Benzimidazole, carbendazim and thiophanate methyl have no effect on yeast (only at very high doses). In contrast, benomyl can be active even at low concentration. Dichlofluanid is the only fungicide whose use requires caution from the point of view of influence on the yeast flora (Santos, 1997).

Triazoles as hexaconazole, penconazole and tetraconazole do not cause any alteration on fermentation kinetics but biocide effects were observed early in their presence at different doses, which causes small slowdowns at the beginning of fermentation (Cabras et al., 1999; Oliva et al., 2000b).

The yeast flora typified carried out in grapes, must and wine obtained by the addition of active dry yeast (LSA) to vegetable material treated with famoxadone fenhexamid, fluquinconazole, kresoxim methyl, trifloxystrobin and quinoxyfen showed the absence of selective effect of all. No differences were observed between species or in the rates of appearance. There is also evidence that the presence of these fungicides do not affect the count levels of yeast in the grapes (even in more adverse treatment conditions) (Table III) or during the fermentation process, taking samples and conducting the count to 1, 5, 12 and 20 days since the start of fermentation (Table IV) (Oliva et al., 2007c).

Unites	Control	Quinoxyfen		Kresoxim		Famoxadone		Trifloxystrobin		Fluquinconazole		Fenhexamid	
		GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP
Log CFU/g	2.5	2.5	3.2	3.7	3.3	3.4	3.7	5.2	3.7	3.3	3.6	3.5	5.9
Log CFU/cm^2	1.9	1.8	3.8	3.0	2.6	2.8	3.0	4.5	3.0	2.6	3.0	2.9	5.2

Table III. Yeast count on the grape surface.

Days	Control	Quinoxyifen		Kresoxim		Famoxadone		Trifloxystrobin		Fluquinconazole		Fenhexamid	
		GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP	GAP	CAP
1	2.6	2.5	4.5	3.7	3.3	3.5	3.8	5.2	3.8	3.3	3.7	3.6	6.0
5	7.8	7.9	9.5	7.6	9.3	7.5	9.5	7.6	9.5	7.4	9.2	7.6	9.5
12	6.9	7.7	9.4	8.1	8.7	8.0	8.7	7.3	9.0	7.6	9.2	8.3	8.5
20	5.6	6.7	8.5	8.8	7.5	8.2	7.8	7.5	7.0	7.2	6.9	8.1	5.2

Table IV. Evolution of yeast during fermentation.

Some authors reported the influence of certain fungicides on the fermentation kinetics of *Saccharomyces cerevisiae*, especially triazole and imidazole.

In trials of new compounds of non-systemic fungicides (fludioxonil and fenilpirrol), some anilinipirimidinas (cyprodinil and pyrimethanil) and new active substances similar in structure to natural strobirulines (azoxystrobin and kresoxim methyl) has been found not to significantly affect development of fermentation in winemaking targets of Airen variety, but more specifically to influence some characteristics of the finished wine (García, 2002).

The influence of pesticides on the malolactic fermentation, not very studied, seems to be not significant. Although, some researchers showed fungicides such as mancozeb, methyl metiram, cymoxamil, dichlofluanid, vinclozolin and iprodione may have depressant effect of this process (Cabras et al., 1999 & 2001).

We have evaluated the *in vitro* inhibitory effect of fungicides the famoxadone, fenhexamid, fluquinconazole, kresoxim methyl, trifloxystrobin and quinoxyfen on growth of *Saccharomyces cerevisiae, Hanseniaspora uvarum, Dekkera bruxellensis*, and *Torulaspora Zygosacharomyces delbrueckii rouxii*, which are yeast flora normally present in initial natural flora of grape and wine. The effect was measured by determining the inhibition halos of different yeast inoculated on the medium GPYA (Oliva et al., 2009b).

The effect of plant protection products tested on different yeast was mixed but generally not apparent inhibition of growth on the surface was appreciated. Only, an inhibitory effect on growth of *H. uvarum* in the presence of kresoxim methyl is clearly shown at a concentration of 400 ppm (concentration resulting from using twice the recommended application) (Figure 4).

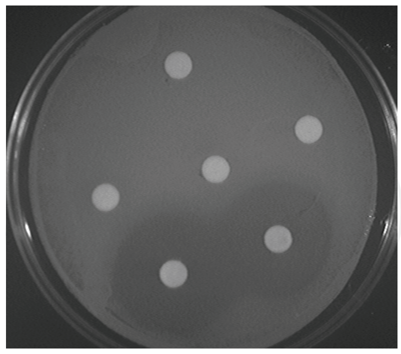

Fig. 4. Inhibition halos of Stroby (400 ppm of kresoxim methyl) on *H. Uvarum*

This effect is manifested by the appearance of a halo of inhibition of 1cm radius. This product does not show any effect on the rest of yeasts included in the study. This same species along with *S. cerevisiae* is slightly inhibited by commercial grade WG Flint (trifloxystrobin 50%) tested at concentration of 300 ppm, though the inhibition zone is less than 1 mm. These results indicate the absence of inhibitory effects of the plant protection products studied on the normal flora in the process of winemaking. The values tested were well above the legislated MRLs by Spain and the EU for wine grapes.

Influence on the aromatic fraction.

Although the descent in the aromas of wine has been studied extensively, various factors such as grape variety, cultural practice, climate, processing, processes, etc., there are few studies on the possible influence of pesticide residues. This is because its presence in grapes can cause a range of effects, often undesirable, and can alter the quality of wine produced by introducing bad tastes and smells into the must and wine.

In this sense, it was found the influence of some fungicides on the aromatic composition of wine, observing significant differences between the levels of major volatile -ethyl acetate, methanol, isobutanol and diethylacetal- and the wines considered classics in terms of phytosanitary treatments -three standard treatments in the area- compared with a control wine (Table V) (Oliva et al., 1999b).

The amounts of methanol are slightly higher than the control wine but double for the classic one. Specifically in the case of fenarimol, the level reaches 107 mg/l (compared to 54.5 in controls), which may be due to the increased activity of the enzyme pectin methyl esterase in his presence. The concentration of isobutanol in wines is due to the assimilation of the aminoacid valine -his predecessor- by yeast and this is a possible cause of the low level found in the classic wine due to their treatments. Remaining levels of other alcohols is normal, compared with the values given by different researchers (Santos et al., 1997; Aubert et al., 1997 & 1998).

For the minor volatile -acids, esters, alcohols and aldehydes-, no significant differences were found between the values of isobutyric and isovaleric acids (bacterial activity rates, poor quality factor). So, both pesticides studied did not affect bacterial activity. The levels of hexanoic acid, octanoic and decanoic acids showed significant differences in the classic wine and also in those treated with fenarimol, vinclozolin and penconazole (the high concentration of hexanoic acid may be due to the increase in the amount of yeast in a more intense fermentation stage). In addition, only significant differences were found for isoamyl acetate, hexyl acetate, ethyl decanoate, ethyl acetate and phenyl ethyl dodecanoato among the fifteen selected esters, though their levels were normal. The latter together with ethyl hexanoate had strong influence on the aromatic profile of young wines.

Aromatic compounds	**Classic**	**Fenarimol**	**Mancozeb**	**Metalaxyl**	**Penconazole**	**Vinclozolin**
Major						
Ethyl acetate	◙					
Diethyl acetal		◙	◙	◙		◙
Methanol		◙				
Isobutanol	◙					
Minoritary						
Hexanoic acid	◙	◙			◙	◙
Heptanoic acid	◙					
Decanoic acid	◙		◙	◙		
Isoamyl acetate		◙				◙
Hexyl acetate	◙			◙	◙	
Ethyl decanoate	◙	◙			◙	
2 Phenyl ethyl acetate	◙				◙	
Ethyl dodecanoate		◙				
3-ol-1-octene	◙	◙	◙	◙	◙	
2-ethyl-1-hexanol	◙	◙				
1-octanol					◙	

Table V. Influence of some fungicides on the aromatic composition of wine.

On the other hand, only 1-octanol and 1-octen-3-ol differed significantly in quantity among the sixteen alcohols analyzed, but if they are present at high concentrations, it can be considered as a defect. Finally, no differences were found in the aldehydes examined and values were normal.

There are references of some pesticides in the family of the triazoles (penconazole, hexaconazole, fluquinconazole, etc.) They produce alterations in the synthesis of sterols and composition of the ester fraction in the aroma of wines (Aubert et al., 1997 & 1998; Oliva et al., 1999b).

Another study on the effects of three fungicide residues (cyprodinil, fludioxonil and pyrimethanil) on the aromatic composition (acids, alcohols and esters) of *Vitis vinifera* white wines (Airén var.) inoculated with three *Saccharomyces cerevisiae.* strains (*syn bayanus, cerevisiae* and *syn uvarum*) show that the addition of the three fungicides at different doses (1 and 5 mg/L) produces significant differences in the acidic fraction of the aroma, especially in the essays inoculated with *Saccharomyces cerevisiae,* although the final contents do not exceed the perception thresholds. The lower quality wines, according to isomeric alcohol content (Z-3-hexen-1-ol and 3-methylthiopropan-1-ol), are those obtained by inoculation with *Saccharomyces cerevisiae* (*syn bayanus*) and addition of cyprodinil. The addition of fungicides in the essays inoculated with *Saccharomyces cerevisiae* (*syn bayanus*) produces an

increase in the ethyl acetate and isoamyl acetate content, which causes a decrease in the sensorial quality of the wine obtained (García et al., 2004).
In the studies already cited -held in La Rioja on Tempranillo grapes-, it was found that the wines made from grapes treated not even reached the minimum of the reference dose (36-350 mg/l) of methanol from the hydrolysis of pectins of the grape but without importance in the aroma though it has because of its toxic effect. All wines met that acetaldehyde, produced in the first phase as an intermediate product of fermentation of yeast metabolism from pyruvate, remained at the lowest possible levels (6-190 mg/l). Wines with dichlofluanid and sub-products of formulations of procymidone and iprodione presented amyl alcohols -formed in yeast- over 180 mg/l (appearance of plant sensations), though there were significant differences between samples. These higher alcohols -more than two carbons- were transferred to the medium and its variations were due to the fermentation process. Their normal ranges are 150-500 mg/l but the effect is negative at levels above 300 mg/l communicating unpleasant herbaceous feelings. The wines of the experience had ethyl acetate levels within the range of acceptable quality, except the sample with dichlofluanid which exceeded the threshold of perception (over 150 mg/l). This ester is the most abundant and high content comes from the accidental development of oxidative yeasts which do not change the volatile acidity or bacteria that oxidize ethanol to acetic conferring elevated hardness and roughness, adhesive and chopped sensations in wine. Nor were there significant differences in the formation of fatty acid ethyl esters (responsible for fruity and floral sensations), though the wine had half the concentration of diclofuanide. No significant differences were found in the total content of acetate and methyl acetate. Not so with the acetates responsible for fruity and flower smell of wines such as isoamyl acetate (principal responsible for the fruity aroma), hexyl acetate and 2-phenylethyl acetate. The wine with dichlofluanid did not contain hexyl acetate or 2-phenylethyl and did not reach the level of perception of isoamyl. The total fatty acid content of more than five carbons were similar to those found in the literature, so these fungicides have little influence over them, except for dichlofluanid that presented the lowest values (Santos, 1997).
Also, the effect of new fungicides (famoxadone, fenhexamid, fluquinconazole, kresoxim methyl, quinoxyfen and trifloxystrobin) residues on the aroma composition of Monastrell red wines shown that all fungicides treatments significantly affect the wine aroma composition, though it does not necessary indicate changes on the sensorial profile as the variation range do not overpass the olfactory threshold of each compound. The most affected group of volatile, in terms of active principle or treatment, were the acetate and acids indicating that all fungicides may have some influence on the yeast activity while alcoholic fermentation takes place. It is important to point out that quinoxyfen and trifloxystrobin do not affect the volatile terpenoids. Kresoxim methyl and fenhexamid active principles have the lowest effect on the aroma composition while fluquinconazole and trifloxystrobin principles were the most reactive (Oliva et al., 2008).

Influence on the acid fraction.

Many oenological properties or aspects are influenced by the acidity of the must and wine. Among them, we may cite the development and metabolism of microorganisms, the wine's color, precipitation of tartaric salts and especially the organoleptic properties.
The acid fraction of wine is also affected by the presence of residues of certain fungicides (fenarimol, metalaxyl, mancozeb and penconazole). In a experiment with different wine-makings -some with treatments, others as control and others from traditional farming-, it

was found that the evolution of total acidity was similar in relative terms during the production for all the tests, but their initial values were different and the final values guaranteed conservation and proper balance in its constitution. The evolution of the volatile acidity was different for the various tests, noting that the final value of volatile acidity decreased more than the control and that there was a dramatic decrease in the case of fenarimol (no justification found) (Oliva et al., 1998b).

Moreover, other studies have shown that residues dichlofluanid at high doses causes acidification of the wine (Oliva et al., 1999c; Santos, 1997). Also, white wines (var. Airén) fermented in the presence of the fungicides azoxystrobin, cyprodinil, fludioxonil, kresoxim methyl, pyrimethanil and quinoxyfen, showed that they cause decreases in the final content of the different acids in most cases (García, 2002).

We have studied the influence of the residues of famoxadone, fenhexamid, fluquinconazole, kresoxim methyl, trifloxystrobin and quinoxyfen (fungicides widely used in vineyards) could have on the content of organic acids (citric, malic, succinic and tartaric) in Monastrell wines. Made two different treatments in the grape -at harvest time and other at critical conditions-, the statistical study indicated that there were significant differences for famoxadone and kresoxim methyl compared for malic acid in GAP, while those treated with fluquinconazole and quinoxyfen for citric acid in critical conditions, fenhexamid fluquinconazole and for succinic acid in both conditions and finally treated with quinoxyfen for tartaric acid in critical conditions. For trials where significant differences were obtained, an increase in the levels of malic and citric acids and a decrease of succinic acid and tartaric acid content were obtained. The final values of the acids studied were typical levels of quality wines, so we can conclude that the residues of fungicides do not affect the final quality of wines despite the differences found (Oliva et al., 2009c).

In studying the effect of the presence of azoxystrobin, quinoxyfen and kresoxim methyl on the final content of organic acids (citric, lactic, succinic and tartaric acids in white Airen wines) inoculated with different strains of *Saccharomyces*, it was observed that the absolute values of organic acids were within the optimum range for quality wines, except tartaric which was too low. From the analytical standpoint, the yeast most affected by the presence of residues of fungicides was *Saccharomyces cerevisiae syn uvarum*, causing declines of citric, lactic and tartaric acid but increases of succinic acid with respect to the control. Therefore and despite the influence of the residues of these fungicides, they do not significantly influence on the organoleptic quality of the finished wine, as they also produce decreases of the final contents of those acids (García, 2002).

Influence on the color and antioxidant activity.

The phenolic compounds are of great importance in oenology, as they are considered the origin of color and astringency (tannins) and have nutritional and pharmacological interest. The factors that influence their content in wine may be soil-climatological, genetic, cultural and oenological ones (presence of pesticide residues).

In relation to color, studying the influence of fenarimol, mancozeb, metalaxyl, vinclozolin and penconazole, there were no significant differences in the intensity of color and tone but does in case of saturation with respect to the control wine in the presence of fenarimol and penconazole (Oliva et al., 1999d).

By studying the influence of the fungicides azoxystrobin, cyprodinil (Switch), fludioxonil (Switch), kresoxim methyl, pyrimethanil and quinoxyfen in the final color of wine, significant differences were found between the vinification of the treated and control grapes.

As it can be seen in the data obtained and presented in Table VI, there were significant differences between the control wine and those made in the presence of residues of fungicides for all parameters except for the total content in anthocyanins and the ortodiphenols. These differences were most pronounced for the wines produced in the presence of pyrimethanil (Fernández et al., 2001).

	Control	**Quinoxyfen (a)**	**Kresoxim (b)**	**Azoxystrobin (c)**	**Swicth* (d)**	**Pyrimethanil (e)**	**DS p ≤ 0.05**
CI	19.27±0.58	15.16±1.26	15.34±0.99	16.15±1.09	15.97±2.75	15.26±0.22	a,b,c,d,e
H	0.45±0.01	0.46±0.01	0.47±0.02	0.47±0.01	0.46±0.01	0.47±0.01	b,c,e
TP	2.45±0.32	1.96±0.08	1.81±0.25	2.12±0.16	1.80±0.16	1.51±0.10	a,b,d,e
FI	48.40±1.05	38.57±4.54	40.50±2.86	42.97±2.56	38.57±4.76	33.80±1.60	a,b,d,e
TPI	83.47±2.45	58.67±4.92	67.97±2.61	71.67±3.70	59.47±7.71	53.10±0.20	a,b,c,d,e
Cat.	257.9±5.1	233.8±24.7	203.2±8.5	221.7±31.9	213.9±23.9	174.0±17.6	b,d,e
Ant.	445.9±29.5	410.4±32.9	385.0±34.6	392.4±40.2	425.2±52.5	421.3±12.6	NS
Tan.	361.9±12.9	309.3±72.5	281.1±17.5	304.2±26.1	298.4±44.1	254.2±8.7	b,e
%M	48.27±3.19	47.52±3.49	47.92±1.50	46.95±0.55	38.23±2.14	41.15±1.84	d,e
%RP	33.58±1.80	38.50±3.44	36.00±0.88	36.44±0.72	44.07±1.61	44.34±2.74	a,d,e
%BP	18.13±1.46	14.65±0.95	16.08±0.73	16.60±1.04	17.69±0.71	14.51±1.05	a,b,e
OD	12.53±3.41	10.28±0.37	9.68±2.24	12.77±1.73	12.31±0.13	11.51±1.51	NS
H_c	19.10±0.10	23.43±1.70	23.93±0.35	23.34±0.74	24.20±2.76	22.10±1.11	a,b,c,d,e,
S	5.26±0.02	4.87±0.54	4.17±0.10	4.33±0.13	4.27±0.50	4.66±0.32	b,c,d,e

CI: Color intensity; **H**: Hue; **TP**: Total polyphenols (g/l galic acid); **FI**: Folín index; **TPI**: Total polyphenols index; **Cat**: Catechins (mg/l D-catechin); **Ant**: Totals anthocyanins (mg/l anthocyanins); **Tan**: Tannins (mg/l Tannic acid); **%M**: Monomers; **%RP**: Red polymers; **%BP**: Brown polymers; **OD**: ortodiphenols (mg/l D-catechin); **H_c**: Hue CIELab; **S**: Saturation; **DS**: Degree of significance; **NS**: Not significant; * Ciprodinil and fludioxonil mix.

Table VI. Influence of some fungicides in the final color of wine

The color intensity is the main element of trial in the visual phase of sensory analysis. The greater or lesser value for this parameter is strongly influenced by the degree of ripeness of the grapes, the maceration time and temperature to which it is made. If we consider that these factors have been the same at all wine-makings, it is conceivable that the presence of studied fungicide residues during the production has helped to reduce the rate of diffusion of the phenolic compounds from the skin into the must during the period of maceration.

The absolute value of total polyphenols index is indicative of the ability of the wine to undergo parenting. We observed great difference between the total polyphenols index for the control wine and those obtained in the presence of pesticide residues in this study.

When we study the phenolic composition of these wines, statistical analysis of the data showed that there were significant differences between the control wine and the remaining ones (Oliva et al., 2005) (Tables VII and VIII).

Regarding the phenolic compounds of low molecular weight (Table VII), it should be noted that there were significant differences between the control wine and those obtained in the presence of residues of fungicides for all compounds studied, except for tyrosol.

In conclusion, the wines made from grapes treated with fungicides presented significant differences for most compounds. Fungicides that influence phenolic compounds were azoxystrobin, kresoxim methyl and pyrimethanil. The minor influence was produced by quinoxyfen. The final contents of all compounds studied were within the values given in the literature.

Compound	Control	Kresoxim (a)	Quinoxyfen (b)	Azoxystrobin (c)	Switch* (d)	Pyrimethanil (e)	DS p ≤ 0.05
Gálic acid	19.08±1.81	13.88±1.16	14.10±3.16	14.08±3.27	12.60±2.19	6.19±0.55	a,b,c,d,e
Tyrosol	27.52±9.29	21.41±6.26	32.05±3.94	24.38±3.33	32.22±8.95	29.78±6.44	-
4-hid.benz. acid	10.34±1.05	5.32±2.31	6.16±2.43	6.87±0.36	4.95±1.06	2.58±1.78	a,b,c,d,e
Vainillic acid	4.23±1.78	4.18±1.26	3.28±1.00	7.93±2.24	3.50±1.88	3.67±1.57	c
Catechin	29.28±11.96	10.25±3.16	12.43±5.57	9.26±3.20	10.12±4.01	14.44±3.88	a,c,d,e
Siringic acid	13.48±1.66	11.07±0.75	10.52±3.25	12.14±2.26	8.63±1.22	7.43±2.53	a,d,e
4-cumaric acid	3.81±2.63	1.65±0.17	2.08±0.40	1.86±0.84	1.66±0.48	2.53±2.09	a,d,e
Ferulic acid	1.39±0.28	4.75±0.62	5.92±0.48	0.89±0.09	3.36±0.76	0.81±0.42	a,b,c,d,e
Tr.-resveratrol	0.52±0.08	1.24±0.34	1.97±0.14	1.74±0.12	1.63±0.14	1.42±0.53	a,b,c,d,e

Table VII. Phenolic compounds of low molecular weight (mg/l) in wines (mean ± DS).

Compound	Control	Kresoxim (a)	Quinixyfen (b)	Azoxystrobin (c)	Switch* (d)	Pyrimethanil (e)	DS p ≤ 0.05
Delfinidin-3-gl.	0.62±0.08	0.66±0.19	0.52±0.07	0.31±0.08	0.82±0.23	0.88±0.35	c
Cianidin-3-gl.	0.26±0.03	0.26±0.06	0.29±0.06	0.26±0.08	0.31±0.05	0.35±0.09	e
Petunidin-3-gl.	1.79±0.18	1.94±0.38	1.78±0.24	0.99±0.32	2.46±0.53	2.60±0.71	c
Peonidin-3-gl.	0.94±0.05	1.11±0.11	0.98±0.13	0.85±0.17	1.26±0.25	1.86±0.42	a,e
Malvidin-3-gl.	13.17±0.64	13.22±1.56	10.83±1.42	7.88±1.63	13.46±1.97	15.91±3.03	a,b,c

Table VIII. Anthocyanins (mg/l) in wines (mean ± DS).

The phenolic composition of wines with different intensity can be altered by the presence of residues of certain fungicides. Thus, in a study conducting microvinifications with spontaneous fermentation by yeast added (LSA) from grapes treated individually with famoxadone, fenhexamid, fluquinconazole, kresoxim methyl, trifloxystrobin and quinoxyfen in critical conditions (same day of harvest), we determined the total amount of anthocyanins, hydroxycinnamic acids, flavonols and trans-resveratrol. No significant differences were found for these compounds among the witnesses with spontaneous fermentation with added yeast. Once the statistical study performed, significant differences were found in the anthocyanin content of grapes treated for famoxadone, fenhexamid and trifloxystrobin; for hidoxicinamic acid in the case of treatments with famoxadone fluquinconazole, kresoxim methyl and trifloxystrobin and differences appeared for trans-resveratrol for all fungicides except quinoxyfen and finally for flavonols in the case of famoxadone and kresoxim methyl. In addition, there was a decrease in the content when these differences occur for the four compounds (Barba et al., 2009a).

When measuring the antioxidant activity of wines, it was found that fungicides did not produce a decrease in the antioxidant activity of wines, both in treatment at pre-harvest

interval as in the case of the most unfavorable conditions (White: 7.19; Treated at pre-harvest interval: 6.45 and Unfavorable: 10.06 Tolox mmol/l). It was noted that the presence of famoxadone, kresoxim methyl and quinoxyfen increased the antioxidant activity directly related to their levels in grapes (Oliva et al., 2009a).
Finally, one can deduce that the presence of some of the studied fungicides may cause a loss of co-pigmentation, lower color stability and decrease in antioxidant capacity. However, the contents analyzed were in the normal range of Monastrell red wines and therefore we should not discourage the use of these fungicides.

2.3 Hygienic-sanitary quality and toxicity for consumer.

If the oenotechnological processes are not performed or done incorrectly, some of the residues in the juice can pass to the end wine and stay for longer or shorter time, leading to poor health and hygiene quality of the finished product.
The presence of residues of fungicides in wine greatly concerns the consumer. Therefore, both the European Union and individual countries are conducting studies to determine transfer factors in the process of making wine and also checking the safety of residues remaining in the finished wine. Currently, there is no established maximum residue limits (MRLs) for specific wines, though there are several countries that provide these values for wine grapes.
We highlight the importance of fungicides as a complement to the establishment of transfer factors, since they can generate harmful effects on humans as toxic. Its bioavailability in the body depends on its toxicological kinetics: absorption, distribution, metabolism and elimination, so it is interesting to study this. These processes are influenced by both external factors related to exposure patterns and the chemical (type of employment, environmental temperature, type of pesticide, frequency, intensity and duration of exposure, etc.) and factors inherent to the individual (age, gender, genetic endowment, health, nutritional status, lifestyle, major route of absorption, etc.) (Fait & Colosio, 1998; Bollinger et al., 2005).
Bioavailability is essentially a pharmaceutical term which refers to the portion of the dose of a drug administered exogenously that reaches the organ or tissue in which it carries out its action. As determining the concentration in tissue is too invasive, the value of the concentration in plasma is accepted. This concept is used to quantify the degree to which a substance is used by the body. Today, the scope of the study of the bioavailability of a xenobiotic has spread to other areas and so there are many studies on nutrient and some pollutants. This value is implied by the ingestion toxicity studies performed in experimental animals in the registration of a substance for pesticide residues, but the substance is applied in pure form -unmixed with food- and has been amply demonstrated in the pharmaceutical field that the presence of food can significantly reduce or even increase the bioavailability of a xenobiotic to the body. Therefore, the study of the bioavailability of a residue embedded in the medium that reaches the body is essential to determine the dose that actually enters it. The results published by our research group have been the first to provide this in food, but we do not include data on them since this was studied for insecticides.

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Fungicides

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Plant and plant products are affected by a large number of plant pathogens among which fungal pathogens. These diseases play a major role in the current deficit of food supply worldwide. Various control strategies were developed to reduce the negative effects of diseases on food, fiber, and forest crops products. For the past fifty years fungicides have played a major role in the increased productivity of several crops in most parts of the world. Although fungicide treatments are a key component of disease management, the emergence of resistance, their introduction into the environment and their toxic effect on human, animal, non-target microorganisms and beneficial organisms has become an important factor in limiting the durability of fungicide effectiveness and usefulness. This book contains 25 chapters on various aspects of fungicide science from efficacy to resistance, toxicology and development of new fungicides that provides a comprehensive and authoritative account for the role of fungicides in modern agriculture.