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Influence of yeast strain on ochratoxin A content during fermentation of white and red must

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Abstract

The aim of this work was to examine whether the yeast strains, responsible for alcoholic fermentation, have an influence on the concentration of ochratoxin A (OTA) in wine. Before the fermentation, OTA was added to musts up to a concentration of about 2 μg/l. OTA content was determined in white and red wines resulting from respective musts and in methanolic extract of the yeast lees (MEL). Data showed a significant reduction of OTA at the end of alcoholic fermentation. However, depending on the yeast strain involved in the fermentation, there was a difference in the content of OTA in the wines. The percentage of OTA removal during the fermentation was between 46.83% and 52.16% in white wine and between 53.21% and 70.13% in red wine. The absence of degradation products suggested an adsorption mechanism. OTA concentration in MEL resulting from red must fermentation was higher than in white. A significant amount of OTA was not recovered either from wine or from MEL.
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Keywords: Ochratoxin A; Yeast strains; Must; Wine polyphenols

1. Introduction

Ochratoxin A (OTA) is a mycotoxin produced by several species of fungi belonging essentially to the *Aspergillus* and *Penicillium* genera. OTA is an isocumarin derivative linked through the carboxy-group to an L-β-phenylalanine (Festas et al., 2000).

The International Agency for Research on Cancer (IARC, 1993) has classified OTA as a probable human carcinogenic substance (Kuiper-Goodman, 1996), and a potent nephrotoxic agent (Plestina, 1996), with teratogenic and immunosuppressive properties ascertained in laboratory animals. The fungi that produce large quantities of OTA belong to the species *Aspergillus ochraceus*, *Aspergillus carbonarius* and *Penicillium verrucosum* (Davis and Diener, 1987; Zimmerli and Dick, 1996; Rousseau and Blateyron, 2002).

These fungi are capable of growing under different conditions of moisture, pH and temperature, and on a variety of foods, such as cereals, coffee, fruit, cocoa, nuts and spices, meat, milk and barley for beer production (Zimmerli and Dick, 1996). The presence of OTA in musts and wines was pointed out for the first time in 1996 in a study realized by Zimmerli and Dick (1996). Numerous research has then confirmed the presence of OTA in musts and wines especially in red and dessert wines (Burdaspal and Legarda, 1999; Cerruti et al., 2000; Festas et al., 2000). The development of the fungi producing OTA essentially depends on the climatic conditions and it is found more frequently in regions with temperate and tropical climate (Zimmerli and Dick, 1996). The presence of OTA in musts and wines is due to fungal contamination of the grapes that may develop both in pre- and post-harvest, or during the phases before the winemaking. The temperature, the moisture, the aeration, the period of infection and the interaction between different fungi are favourable factors for mycotoxin development (Codex Alimentarius Commission, 1997; Scudamore et al., 1999). The presence of OTA on grapes is mainly due to growth of *A. carbonarius* (Rousseau and Blateyron, 2002). Researches on the frequency and concentration of OTA in samples of food and human blood

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indicate that numerous food products are contaminated (IARC, 1993; Breitholtz-Emanuelsson et al., 1994). Recently, the EU has established a maximum allowable OTA concentration of 2 ppb for wines starting from the vintage of 2005 (Gazzetta ufficiale dell'Unione europea, 2005), reserving the possibility of lowering this limit following the new technological and toxicological research.

The recent investigations are, therefore, concentrated on the study of possible mechanisms of degradation and decontamination of OTA in must and wine (Castellari et al., 2001; Dumeau and Trionè, 2002; Rousseau and Blateyron, 2002; Bejaoui et al., 2004; Garcia Moruno et al., 2005). A variety of fining agents was evaluated in relation to the capabilities to remove OTA in wine. Castellari et al. (2001) found that potassium caseinate and activated carbon showed high adsorption capacity for OTA. Treatment of red wine containing OTA added with active dry yeast or with yeast lees obtained from alcoholic fermentation was also evaluated (Garcia Moruno et al., 2005). Other studies suggest that fungi of *Aspergillus* genus, degrade some aflatoxins, possibly through fungal peroxidases, and that fermentation with yeast strains can destroy the patulin and rubratoxin B (Lopez-Garcia and Park, 1998). The decomposition of trichotecin and isotrichotecin was also investigated during fermentation of grape juice (Flesch and Voight-Scheuerman, 1994). Concerning the mechanism of decomposition, the authors suggested an enzyme activity. In another experiment, Scott et al. (1995) fermented a wort, containing added OTA, with three different yeast strains of *Saccharomyces* spp. After 8 days OTA decreased by 13%. It was found that the yeasts adsorbed 21% of the OTA removal. In a recent work, Bejaoui et al. (2004) demonstrate that oenological *Saccharomyces* yeast strains were able to remove OTA from different synthetic media which initially had OTA added, and also from red grape juice. The action carried out by non-*Saccharomyces* yeasts on the OTA present in must, during fermentation and the effect of the nature of must (white or red) is not known yet.

The aim of the present study is to investigate whether oenological *Saccharomyces* and non-*Saccharomyces* yeast strains can reduce OTA content present both in white must (WM) and red must (RM) during the fermentation process.

2. Materials and methods

2.1. Strains of yeast, preparation and analysis of the musts

The yeast strains used in this study and their origin are reported in Table 1. In order to verify an eventual interaction between yeast strains and the medium, the fermentation was carried out on two natural musts (WM and RM). The WM was obtained, from *Trebbiano Toscano* and *Malvasia del Lazio* grapes. Grapes were crushed and pressed using a basket press, with a maximum pressure of 400 psi. The clarification of must was carried out, adding bentonite (100 g/hl), gelatin (10 g/hl) and kept at the controlled temperature of 8 °C for 48 h and then racked. The RM was obtained by diluting concentrated RM from Primitivo grapes with RM from the same Primitivo grapes. The musts were sterile-filtered through cellulose membrane filters with 0.2 μm pore size (Sartorius AG W-3400, Goettingen, Germany). The musts had OTA (4-6912, Supelco, Bellefonte, Pennsylvania, USA) added up to a concentration of about 2 μg/l confirmed by analysis. The determination of OTA was carried out by using the HPLC method outline by Visconti et al. (1999). Analysis was performed with a high-pressure liquid chromatograph (HP 1100 Hewlett-Packard, Waldbronn, Germany) equipped with pump (HP G1311A Hewlett-Packard, Waldbronn, Germany), autosampler (HP G1313A Hewlett-Packard, Waldbronn, Germany) and fluorescence detector (HP G1321A Hewlett-Packard, Waldbronn, Germany). Soluble solid (reducing sugars) were measured as Brix°, the titratable acidity and pH were determined according to standard methods (Office International de la Vigne et du Vin (OIV), 1991), total polyphenol content and total anthocyanins were determined using the Di Stefano and Cravero method (1991). The results are shown in Table 2.

Table 1
Yeast strains used

Yeast strain	Identification code	Source	Species code
Saccharomyces cerevisiae	S10c	ISE[a]	Scer
Saccharomyces cerevisiae × *bayanus*	S6u	ISE	Suva
Saccharomyces bayanus	S1b	ISE	Sbay
Kloeckera apiculata	IMIAT-111	UT[b]	Kl
Torulaspora delbrueckii	IMIAT-70	UT	T
Schizosaccharomyces pombe	Schp.3	ISE	Schp
Candida pulcherima	IMIAT-179	UT	C
Saccharomycodes ludwigii	Slud-1	ISE	Slud

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Table 2
Composition of the musts used

	White must	Red must
Brix°[a]	22.28	20.79
TA (g/l)[b]	7.13	7.05
pH	3.18	3.10
OTA (μg/l)	1.97	2.26
Total polyphenols (mg/l)	175	1119
Total anthocyanins (mg/l)	—	321

The values represent the average of duplicate determinations.
[a]Soluble solids measured as Brix°.
[b]Titratable acidity (TA) expressed in g/l of tartaric acid.

2.2. Fermentation condition

For every yeast strain, four independent replications were done for each of the two musts. Fermentations were conducted under aseptic conditions in 1.0 l conical flasks containing 500 ml of must. Musts were inoculated with pure cultures of yeast, pregrown in the same sterile juice for 72 h at 28 °C, followed by 12 h temperature equilibration at 20 °C. At the time of inoculation, total and viable cell counts were performed. After inoculation, the final concentration of yeast cells in the musts was about 10^6 cell/ml. Fermentations of the inoculated musts were carried out at a controlled temperature of 20 °C. They were monitored by weight loss caused by CO_2 production.

2.3. Treatment of the wines and yeast lees

At the end of fermentation the wines were immediately separated from the lees (yeast sediment) by centrifugation at 3000*g* for 15 min at 20 °C. The remaining yeast lees were washed with distilled water and centrifuged at 3000*g* for 15 min at 20 °C. After elimination of the supernatant, they were resuspended in 30 ml of absolute methanol (Carlo Erba Reagenti, Rodano, MI, Italy) for 24 h to extract OTA. After centrifugation at 3000*g* for 15 min at 20 °C, the methanolic supernatants (methanolic extract of the yeast lees, MEL) were separated, collected in 50-ml round-bottom flask and evaporated to dryness by rotary evaporator. The dry residues were resuspended in a mobile phase, made up of 49.5% acetonitrile:49.5% deionized water:1.0% glacial acetic acid (v/v/v), just before the chromatographic analysis (Visconti et al., 1999) to determine OTA concentration.

2.4. Experimental design and statistical analysis

The experimental design comprised the use of eight yeast strains and two musts for a total of 16 assays each replicated four times for a total of 64 samples. The statistical analysis of the OTA content determined both in wines and in MEL was carried out with the single-factor analysis of variance (ANOVA), the Tukey test and graphic representation by using the box plot. For the data analyses, StatSoft statistical package (version 5.1, StatSoft Inc., Tulsa, Oklahoma, USA) was used.

3. Results and discussion

3.1. Alcoholic fermentation in the presence of OTA

According to the alcoholic fermentation rate (Table 3) the time contact between OTA and yeast cells was, with exception of *Kloeckera apiculata* and *Candida pulcherrima*, of the same duration both for WM and RM. The biomass produced in WM and RM at the end fermentation was not significantly different, with exception of *Schiz. pombe*. Whatever the must and the yeast strains, OTA was lowered in all the wines (Table 4). The percentage of OTA removal during the fermentation was between 46.83% and 52.16% in white wine and between 53.21% and 70.13% in red wine, depending on the yeast strain used (Fig. 1).

The data, therefore, pointed out that the capability of the yeast to decrease the OTA content was higher in RM wines. The OTA concentration (μg/l) found in MEL from the RM wines was higher than that found from the WM wines (Table 4). The percentage of OTA found in MEL derived from WM and RM was between 17.16% and 21.54% and between 22.40% and 30.30% of the initial concentration, respectively. Therefore, the yeasts fermenting the RM showed a higher capability to adsorb the OTA. However, in all samples, a considerable quota of OTA was not recovered, neither MEL nor in the wines (Table 4). HPLC chromatograms of wines and MEL pointed out the absence of degradation products (results not shown). This suggested that adsorption rather than biodegradation might be involved. Therefore, we suppose that the OTA not recovered might still be bound to the surface of the yeast and that methanolic extraction of the yeast lees was incomplete. The adsorption of OTA can probably be carried out by cell walls. According to Huwig et al. (2001) the cell walls harbouring polysaccharides (glucan, mannan), proteins and lipids exhibit numerous different and easy accessible adsorption sites including different adsorption mechanisms, e.g. hydrogen bonding, ionic or hydrophobic interaction. The adsorption mechanism can be unspecific as it has been shown for yeast killer toxins bound by polysaccharides and not by proteins, fatty acids, cellulose or glycogen (Radler and Schitt, 1987).

Independent from the adsorption mechanism, the main fact is that alcoholic fermentation causes a reduction of OTA content initially present in the WM and RM. In fact, there was an average reduction of OTA content in the white and red wines of about 49% and 62%, respectively.

3.2. Effects of must on the reduction of OTA during the alcoholic fermentation

Fig. 2, relative to OTA content in both wines (Table 4), shows that in wines from RM the yeast strains *S. cerevisiae*, *S. bayanus* var. *uvarum*, *S. bayanus* and *Schiz. pombe* had a

Table 3
Fermentation profiles of yeast strains in white must and red must

Yeast strain	Weight loss (g/500 ml)						Biomass (g/500 ml)	SD
	Day 3	Day 10	Day 20	Day 25	Day 30	Day 36		
White must								
Scer	12	33	47	49	50	51	5.09	(0.18)
Suva	12	33	48	50	51	51	4.80	(0.29)
Sbay	10	31	46	49	50	51	5.00	(0.41)
Kl	2	10	16	22	—	—	4.55	(0.57)
T	5	15	27	33	37	40	5.26	(0.61)
Schp	6	22	33	38	41	43	7.52	(1.34)
C	1	12	28	28	—	—	6.04	(0.81)
Slud	9	25	37	40	43	45	5.20	(0.52)
Red must								
Scer	15	38	47	48	48	48	4.49	(0.37)
Suva	17	37	45	46	44	44	4.95	(0.11)
Sbay	13	36	45	46	47	47	4.26	(0.45)
Kl	2	13	27	27	—	—	3.76	(0.26)
T	4	14	31	41	46	46	4.58	(0.88)
Schp	7	24	31	35	38	39	9.77	(0.57)
C	1	6	25	27	—	—	6.03	(1.08)
Slud	6	19	31	36	39	41	3.89	(0.65)

Fermentation rate was measured as (CO_2 evolution) weight loss (g/500 ml). Values represent the averages of four replicates. Replicate samples varied by less than 10%. Biomass quantity (g/500 ml) produced at the end fermentation represents the averages of four replicates.
SD, standard deviation.

Table 4
OTA content, in white and red wines, in MEL, after fermentation from inoculated musts, and OTA not recovered obtained from the difference between the initial amount of OTA and the amount found in wines and in MEL

Yeast strain	OTA wine		OTA MEL		OTA not recovered	
	μg/l	SD	μg/l	SD	μg/l	SD
White wines (1.97 μg/l initial content)						
Scer	1.05	0.09	0.39	0.04	0.53	0.06
Suva	0.95	0.08	0.41	0.01	0.61	0.08
Sbay	1.05	0.06	0.37	0.03	0.55	0.04
Kl	1.05	0.06	0.34	0.02	0.58	0.08
T	0.94	0.12	0.40	0.03	0.63	0.14
Schp	1.03	0.07	0.32	0.13	0.61	0.18
C	0.95	0.15	0.38	0.10	0.64	0.04
Slud	0.97	0.11	0.42	0.09	0.57	0.09
Red wines (2.26 μg/l initial content)						
Scer	0.77	0.09	0.69	0.13	0.81	0.14
Suva	0.80	0.08	0.63	0.12	0.83	0.13
Sbay	0.88	0.05	0.67	0.05	0.71	0.05
Kl	1.06	0.05	0.61	0.01	0.59	0.05
T	0.95	0.08	0.51	0.06	0.81	0.12
Schp	0.68	0.02	0.45	0.04	1.13	0.06
C	0.91	0.04	0.61	0.03	0.74	0.02
Slud	0.97	0.10	0.55	0.10	0.74	0.15

The values refer to the arithmetic average of four replications for each yeast tested.
SD, standard deviation.

higher capacity for reducing the content of OTA. The other yeast strains did not seem to be influenced by the type of must, since there were no significant variations in the reduction of OTA in white and red wines. Therefore, an ability of decreasing the OTA content during the fermentation process was evident for all the yeast strains tested. However, it is clear that only for some yeasts the must seemed to affect the OTA decrease. It was shown in previous research (Garcia Moruno et al., 2005) that yeast lees, obtained from alcoholic fermentation of WM, showed

a greater adsorption capacity of OTA content in red wine compare to yeast lees obtained from alcoholic fermentation of RM. The differences found in the treatments with white and red lees were supposed to be due to competition between phenolic compounds and OTA for the same binding site on the cell surface. Bejaoui et al. (2004) in recent work tested the ability to remove OTA from yeast peptone glucose, synthetic grape juice medium and natural grape juice, by using dead cells (heat- and acid-treated) and viable cells of oenological *Saccharomyces*. The authors showed that dead cells enhanced significantly OTA removal compared to the same viable cells. They supposed that the phenomenon depended on enhancement of adsorption sites due to heat or acid cells treatment. In both works, the contact time of yeast cells with the medium and OTA was short compared to the usual time of alcoholic fermentations. However, an adsorption mechanism was involved. Our results showed that besides an adsorption phenomenon between OTA and yeast cells, an independent interaction between OTA and phenolic compounds, or between these and other metabolic compounds, produced by yeast was possible. These metabolites interacting with phenolic compounds might probably increase the surfaces for OTA binding. Escot et al. (2001) have demonstrated that the phenolic compounds interact with the mannoproteins produced by yeasts and that the amount of mannoproteins released during fermentation depends on yeast strain and must type. Other researchers have shown (Grünkemeier, 1990; Bauer, 1994) that yeast or yeast cell walls can be used as adsorbents for OTA and other mycotoxins, and that this depends on yeast macromolecules, such as mannoproteins. Therefore, during alcoholic fermentation viable yeast cells release a higher amount of mannoproteins into the surrounding medium than the dead yeast cells isolated at the end of fermentation and then used to remove OTA in wine. It is probable that mannoproteins interact with polyphenol compounds of RM and could raise the ability to adsorb OTA. Direct interaction between OTA and phenolic compounds could be due to amino group ionization of the OTA molecule in the acid media, like must. This might react with quinones or other compounds derived by enzymatic and non-enzymatic oxidation of polyphenols (particularly anthocyanins in RM) during the fermentation process, and this OTA could be no more detectable in the supernatant (hypothesis to demonstrate in future). Obviously, the

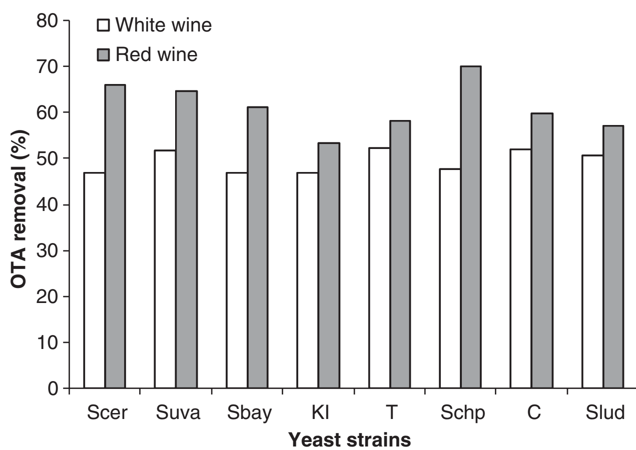

Fig. 1. Percentage values of OTA removal by yeast strains at the end of fermentation in white wine and red wine.

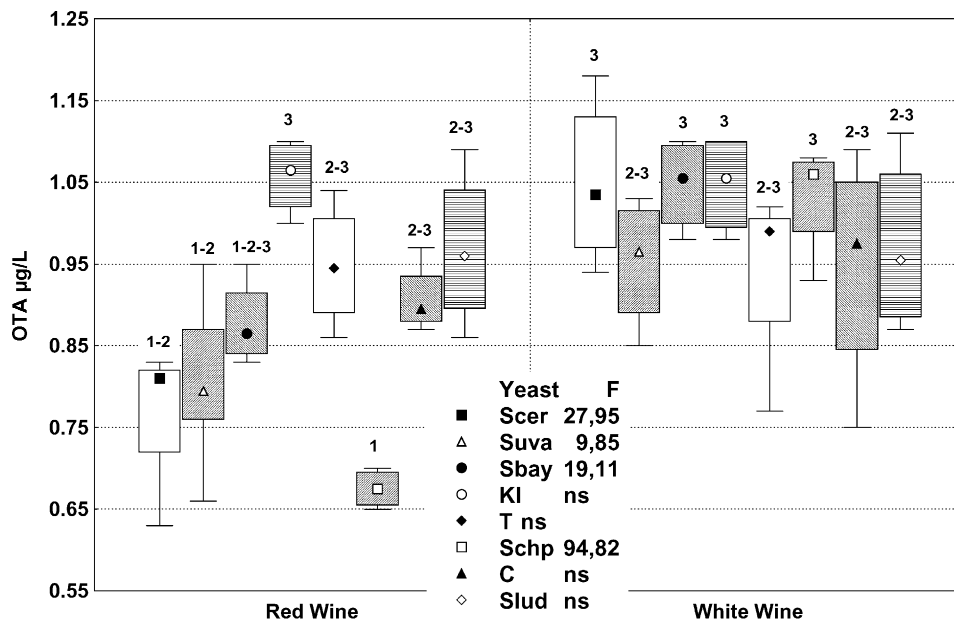

Fig. 2. Box plot representing OTA content (µg/l) in red and white wines obtained from the inoculated musts. Box whiskers indicate extreme values, the box bottom and top define the first and third data quartiles, the symbol, corresponding to the yeast used, indicates the median. In the description, the *F*-values indicate significant differences at $P \leqslant 0.01$ and ns indicates not significant differences, respectively, found by ANOVA. Different numbers denote a significant difference at $P < 0.05$ (Tukey test). There is no difference between samples with the same number.

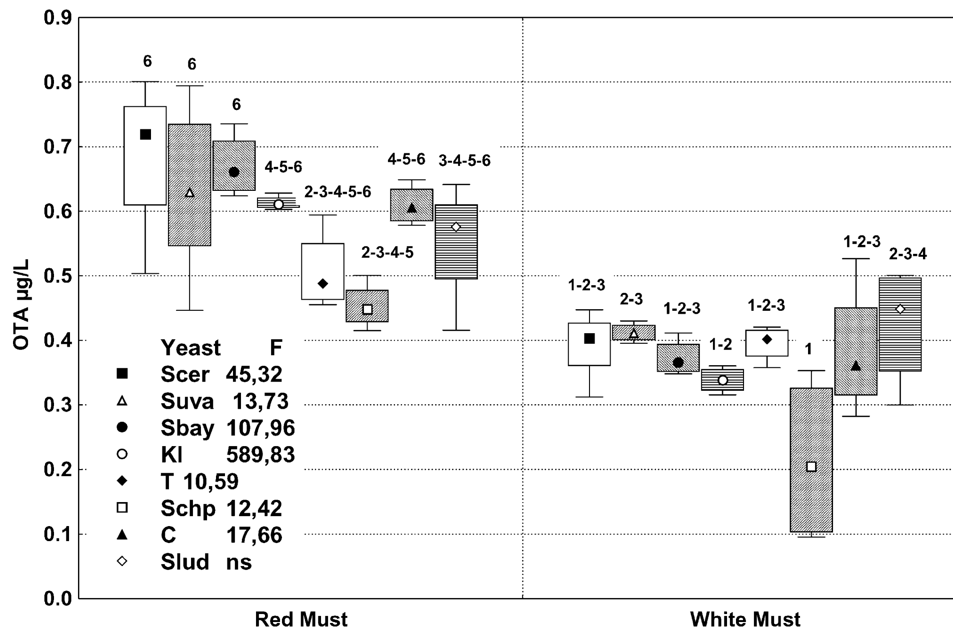

Fig. 3. Box plot representing, for each sample, the OTA content (µg/l) in MEL derived from red and white inoculated musts. Box whiskers indicate extreme values, the box bottom and top define the first and third data quartiles, the symbol, corresponding to the yeast used, indicates the median. In the description, the *F*-values indicate significant differences at $P \leqslant 0.05$ and ns indicates not significant differences, found by ANOVA. Different numbers denote a significant difference at $P < 0.05$ (Tukey test). There is no difference between samples with the same number.

phenolic compounds play an important role in OTA removal. This was also confirmed by the variability to degrade OTA in white and red wine. The interaction between OTA, yeast and phenolic compounds will be studied in future experiments.

3.3. OTA determined in MEL

In all yeast strains tested, with the exception of *S. ludwigii*, OTA found in MEL, derived from the fermentations of RM, results as being significantly different from that determined in the MEL derived from the fermentations of WM (Fig. 3). In the MEL derived from the fermentations of RM with *S. cerevisiae*, *S. bayanus* var. *uvarum* and *S. bayanus*, the largest amount of OTA was found, confirming the capability of these strains to further reduce the OTA content initially present in the must (Fig. 2). According to the Tukey test, the values of OTA found in the respective MEL were not significantly different. In this case, too, the substrate of fermentation seemed to influence the capability of the adsorption of OTA on the lees.

In conclusion, the experiment conducted demonstrated that at the end of the alcoholic fermentation by different yeast strains, the initial OTA content present in the must (RM and WM) decreased in a significant way. This phenomenon was more evident in red wines, in particular derived from fermentation conducted with *S. cerevisiae*, *S. bayanus* var. *uvarum*, *S. bayanus* and *Schiz. pombe*. A significant percentage of OTA eliminated from the fermentation substrate has been found in MEL. The concentration of OTA in MEL of wines fermented with *S. cerevisiae*, *S. bayanus* var. *uvarum* and *S. bayanus*, showed that these yeast strains present a higher capability of adsorbing the OTA in the yeast lees, especially MEL derived from MR.

Since the degradation products were not found, we conclude that OTA was not degraded by yeast strains. The OTA removal, during fermentation process, might be a cell-binding phenomenon; therefore, the non-recoverable OTA might still be bound to the yeast surface. Considering the initial concentration of OTA in must and the average reduction by fermentation, it is possible to predict if the final concentration in wine is within the limits of the law.

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