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*Slobodan M. Jović*
*Aleksandar V. Petrović*
*Nebojša R. Marković*

Faculty of Agriculture, Nemanjina 6, 11080 Zemun, Serbia

# MYCOTOXINS IN WINE WITH SPECIAL ATTENTION ON OCHRATOXIN A

ABSTRACT: Wine quality is a complex, multi — layered conception consisting of numerous factors such as sensory characteristics, chemical composition, legislation, market — consumer, with hygienic — toxicological factor being of special importance due to growing demands for health safe foods. This paper shows the results of studies carried out up till now concerning the mycotoxins in wine (Aflatoxins, Trichothecens, Patulin), with special attention paid to ochratoxin A, most frequently present in grapes, must and wine, and to the influence of certain technological operations and processes during wine making. Due to its high toxicity, the presence of ochratoxin A has been limited to 2 μg/l by EU EG regulation 123/2005.

KEY WORDS: wine quality, mycotoxins, Ochratoxin A

## INTRODUCTION

During the last fifteen years, there has been a constant interest in the quality of food products, so wine is also often the subject of expert — scientific discussions. Wine quality is a very complex and multy — layered concept, considering the numerous factors it comprehends. Total wine quality, as an integral concept represents overall of individual qualities, and their exact determination is the only way for their comprehension and evaluation. It could be said with certainty that wine quality factors are its sensory characteristics, chemical composition, hygienic — toxicological, market — consumer factors, as well as legislation. Some of these factors, such as chemical composition and hygienic — toxicological represent so called "internal" quality, while sensory characteristics represent "external" quality perceived by senses. Apart from these, which can not be separated from wine, there are factors that do not participate directly in the structure of total wine quality, but show an indirect influence (Jovic, S., 1993; Jovic, S., Kovac, V., 1995).

With emphasised demands for health — safe food, meaning the absence of undesirable compounds and pollution agents, hygienic — toxicological quality factor is gaining importance. Some pollution agents get into wine from the outside (heavy metals, pesticides, radionucleides), while certain undesirable compounds are a consequence of activity of yeasts, bacteria or mould on grapes or in must and wine (biogenic amines, ethylcarbamate, higher alcohols, methanol and mycotoxins). Therefore, it is a constant obligation of wine producer to enrich wine with compounds that have positive influence on health and prevent formation of the harmful ones, by applying current scientific knowledge. Having this in mind, it is also a constant task of oenology to establish possible presence of certain mycotoxins in wine, stop their occurence in wine and find the way to reduce their quantity below the allowed maximum. This is, at the same time, an effort to ensure the wine keeps its epithet "The healthiest and most hygienic drink" given by the famous French chemist and biologist Luis Pasteur.

## BASIC FACTS ON MYCOTOXINS

Mycotoxins are secondary products of mould metabolism that are toxic for humans, animals and plants in very small quantities, but not for micro — organisms that produced them. They are mainly low molecule, thermo — stabile compounds which, by their chemical disposition, belong to various groups of compounds. Some mycotoxins remain in mould mycelium as endotoxins, while others, such as ectotoxins, get discharged into the surrounding environment. Unlike toxins of higher plants, for a long time there has been little knowledge about mycotoxins as toxic products of hiphomycetes. Although, as far as 1980, Japanese pathologists had the knowledge about certain species of *Penicillium* that appeared on rice and produced harmful for humans. It was not until the discovery of aflatoxins in 1960. that the studies on mycotoxins started to get into full swing. Certain mycotoxins differ, among other things, in demonstrating acute or chronic toxicity. The chronic one shows carcinogenic, mutagene and teratologic actions. Diseases caused by mycotoxins are called mycotoxicosis (Müller, 1983). From about 300 known mycotoxins the most important are: aflatoxins, citrinin, patulin, ochratoxin A, fumonisin, trichohecene (dezoxynivalenol, nivalenol, $T_2$ — toxin, $HT_2$ — toxin) and zearalenone.

Mycotoxins can appear on plants and their yield, prior to harvest or after it, if the storage conditions are bad. Foodstuffs can get contaminated by mycotoxins during different phases of food chain if mould of species *Aspergillus*, *Fusarium* and *Penicillium* appear and multiply, as they are considered to be the main producers of mycotoxins. Some estimates show that as much as 25% of world products of plant and animal origin is contaminated with mould so, apart from the economic losses, there is always a danger of intoxication with mycotoxins.

First studies on mycotoxins in oenology dealt with aflatoxins at first, and then with patulin and trichotecene, but, since 1996, they have been focused mainly on ochratoxin.

## FORMER STUDIES ON MYCOTOXINS IN OENOLOGY

Aflatoxins were the first mycotoxins in oenology to be studied, most probably because they are the most toxic ones, and their analytic proving methods were the first to be developed. Aflatoxins ($B_1$, $B_2$, $G_1$ and $G_2$) are produced exclusively by mould *Aspergillus flavus* and *Aspergillus parasiticus* types which require higher temperature and air humidity for their development. Aflatoxin $B_1$ is the main toxin which often appears together with $B_2$ or those from G group. The food most frequently contaminated by aflatoxins is: peanuts, hazelnuts, almonds, pistachios, wallnuts, dried fruits and spices. If stock — feed contains these toxins, aflatoxin $M_1$ can be found in milk and milk products. Aflatoxin $B_1$ is one of the strongest carcinogenes which causes liver tumor. In comparison to $B_1$, aflatoxin $M_1$ is somewhat less toxic and has far less influence on appearance of cancer.

At first, Schuller et al. (1967) made tests with 33 types of wine and aflatoxin content that was under 1 μg/l was found in two samples, but according to Franc and Eyrich (1968) some other compounds, with their fluorescence can simulate the presence of aflatoxins. Radler and Theis (1972) analised 215 samples of mouldy grapes and did not find *Aspergillus flavus*, mould that produces this toxin in any of them. Drawert and Barton (1974) did not find aflatoxins in 17 white wines from Rheinpfalz, neither did Lemperle et al. (1975) in 150 samples of Baden wines from 15 different vintages.

According to the former researches, aflatoxins have not been found on healthy or mouldy grapes, nor on grapes with noble rot (*Botrytis cinerea*). Also, their presence has not been proved in wines of all quality categories, in marc or enzyme preparations used in wine technology.

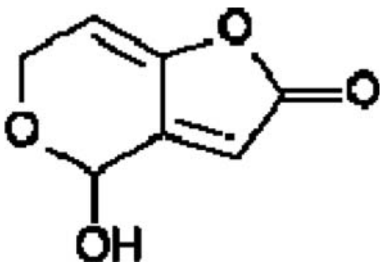
Patulin is a toxin that originates as a secondary product of metabolism of mould *Byssochlamys nivea*, *B. fulva*, *Penicillium urticae*, *P. expansum*, *P. chrysogenum*, *Aspergillus clavatus*, *A. terreus* and others. These moulds can be found on cereals, in bakery and meat products. Patulin can be found, relatively often, on fruit infected with *P. expansum* (Brown rot), and, unless rotten fruit is removed, it can be found in fruit mash and juices. It can be found mainly in apple juice, which results in poor quality and bad technology in juice production.

In must, obtained from grapes infected with mould, originating from Canada, Scott et al. (1977) have found patulin in quantities 30 μg/l to 4,5 mg/l, while patulin was not present in wines obtained from the sam must. Altmayer et. al. (1982) have tested 64 samples of must from Rheinpfalz, and 22% of the samles contained patulin in quantities below 50 μg/l, while 16% of the samples contained 50 μg/l and more, with the highest value being 280 μg/. Mortimer et al. (1985) have tested 13 samples of white and red grape juice and no contamination with Patulin was found at detection limit of 5 μg/l. During alcohol fermentation, decomposition of Patulin occurs faster at higher temperatures. So, Woller and Mayerus (1986) established that fermentation at 17°C completely decomposes 1000 μg/l of Patulin in three days, at 13°C in seven days, while at 8°C two weeks were necessary for its complete decomposition. Patulin has inhibitory effect on numerous types of microorganisms, which is why it was studied as antibiotic at first, but due to its high acute toxicity for humans, animals and plants it is classified as mycotoxin. It does not show carcinogenic effect, but damages DNA and inactivates enzymes with SH group.

Chemically, Patulin is anhidro-3-hydroxymethilen-tetrahydro-1,4-piron-2--carboxyl acid. At pH between 3,0 and 6,5 it is stable, and at higher pH values lactonic ring opens and toxicity disappears. Having in mind its decomposition during alcohol fermentation, there is very small possibility of its occurrence in wine.

Trichothecenes include about 100 mycotoxins produced by *Fusarium.* These toxins have very wide spectrum of biological effects: phytotoxic, insecticide, fungicide, antiviral and cytotoxic, and toxicity is demonstrated by inhibition of protein biosynthesis in cells of mammals. Poisoning with trichothecens provokes damage of bone marrow, small intestine, lymph system, heart muscle etc.

It could be said that the presence of these toxins on grapes, in must and wine has not been systematically analised. *Trichothecium roseum* is another mould that causes bitter rot on apples, while on grapes, it appears as a secondary infection, after *Botrytis cinerea*. It is able to produce mycotoxins trichothecenes, trichotecolon, and rosenon (F l e s c h et al., 1986). Already in quantity of 2,1 mg/l trichothecene can inhibit alcohol fermentation during which it remains unchanged (S c h w e n k and A l t m a y e r, 1985).

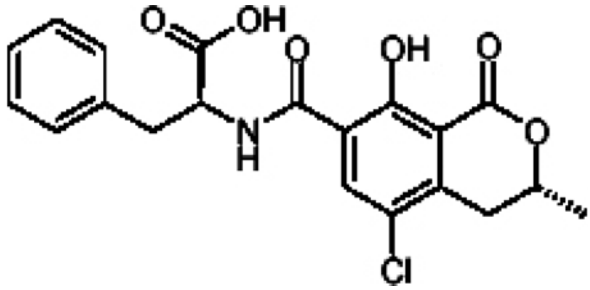



Patulin

## OCHRATOXINS

Ochratoxins isolated in South Africa in 1965. were cyclic penthadicetides, i.e. derivatives of dihydroxyisocumarine tied to L-phenylalanine. Apart from ochratoxin A (OTA), which is the most important due to its toxicity, there is ochratoxin B, derivative of ochratoxin A that does not contain chlorine, as well as ochratoxin C which is ethylester of ochratoxin A. Bruto formula OTA is $C_{20}H_{18}ClNO_6$, molecul substance 403,8, chemical term (7-(L-β-phenyl-alanyl-carbonil)-carboxyl-5-chloro-8-xydroxi -3,4-dihydro-3R-methylisocumarin).

Ochratoxin A is produced by *Aspergillus* and *Penicillium* species of mould. Production of this mycotoxin was initially established with species *Aspergillus ochraceus*, later or also with *A. melleus*, *A. muricatus*, *A. petrakii*, *A. sclerotio-*



Structure formula of OTA

*rum* and *A. sulphureus*, *A. albertensis* and *A. alliaceus* (V a r g a et al., 2001). *A. glaucus*, *A. sydowii* and *A. repens* have also been identified as OTA producers, by the same authors. Furthermore, it has been established that OTA is a metabolite of certain species of *Nigri* section such as *A. niger*, *A. carbonarius*, *A. awamori* and *A. foetidus*. Some species of *Penicillium* also produce OTA, of which the best known are *P. verrucosum* and *P. veridicatum*. The stated moulds have been isolated from various cereals and their products, and can be found in coffee, meat, cheeses, beer, fruit juices, grape juice and wine.

The tests have confirmed that OTA has nephrotoxic and hepatotoxic effect causing kidney and liver cancer. OTA is also connected to the already known Balkan endemic nephrophathy and appearance of tumors in human urinary tract. This mycotoxin has genotoxic, immunotoxic, teratogenous and neurotoxic effect. International Agency for Cancer Research (IARC) has placed OTA into $B_2$ group, i.e. among substances potentially carcinogenic for humans. Taking into consideration that, according to the evaluations of Codex Alimentarius Commission (1998) about 15% of total daily OTA quantity enters organism through wine, EU Regulations Commission (EC 123/2005) has limited maximum OTA content in wine and grape juice to 2,0 ng/ml.

Ochratoxin A on grapes, in must and wine in the first 20 samples of sultanas from Retail network were analysed in Great Britain (M a f f, 1997) and 88% of the samples contained OTA ranging from 0,2 to 53,6 μg/kg. In France, a research was carried out with 373 samples of grape cultivars Carignan, Syrah, Suvignon and Muscat with regard to the appearance of mould after bunch closure, during veraison and during harvest (R o u s s e a u, 2001). Mould contamination of grapes was growing with the development of grapes, and during veraison only 10% of mould produced OTA, and 47% in full ripeness. Out of the identified OTA that produced moulds, 96% belonged to *Aspergillus* species, of which 95% belonged to *A. carbonarius* and 1% to *A. niger*, while about 4% was of *Penicillium* species.

Z i m m e r l i and D i c k (1995) were the first ones to report the existence of OTA in wine and the found quantities ranged from 10—20 ng/l. In their next paper published in 1996, the authors reported the results of more detailed research on OTA in wine. They analysed 118 table wines and 15 special wines sampled from Swiss retail network and found OTA content of about 3 to 388 ng/l, of which the average value for five dessert wines was 337 ng/l.

From the obtained results, it can be concluded that bigger contamination was found in red wines, particularly those originating from South Europe and North Africa. Mayerus and Ottender (1996) analysed 114 wines and found median of OTA concentration of 7 ng/l for white and 200 ng/l for red wine. The highest content of 1850 ng/l was found in red wine from Algeria which is in agreement with the opinion of former researchers that more frequent appearances of OTA and higher concentrations are typical for wines from Mediterranean basin. In Great Britain 10 samples of red wine have been analysed (Maff, 1997) and OTA concentrations were more than 20 ng/l in all samples, the highest level of concentration being 1100 ng/l.

According to the data from literature, mean OTA values in red wines ranged from 0,039 μg/l (Zimmerli and Dick, 1996) to 1802 μg/l (Cerruti et. al., 2000) and were very close to those in rose wines with range from 0,025 μg/l (Zimmerli and Dick, 1996) to 1348 μg/l (Cerruti et al., 2000). These average values are somewhat higher than those found for white wines that ranged from 0,011 μg/l (Zimmerli and Dick, 1996) to 0,535 μg/l (Visconti et al., 1999). Mayerus et al. (2000) are of opinion that different OTA concentrations in white, rose and red wines are caused by the method used in the production of these wines.

Domijan and Perajica (2005) analysed OTA content in 7 white and 7 red wines produced in Croatia. In red wines, OTA level ranged from 12—47 ng/l and in white wines, from 15—22 ng/l. Three white wines from the north (continental Croatia) did not contain OTA, while all those originating from the southern Croatia did contain OTA.

As for OTA content in must, i. e. grape juice, Zimmerli and Dick (1996) analysed 8 samples of red and 3 samples of white commercial must and established median of OTA content of 116 ng/l for all samples, while for the samples of red juice only, it was 235 ng/l. Mayers and Ottender (1996) analysed 20 samples of grape juice and established median of 1800 ng/l for red grape juice, while the highest content was 4700 ng/l. Abrunhosa et al. (2005), in eight independent experiments, after crushing of grapes, found OTA content in juice that was 59% ± 14 from the total concentration existing on grapes.

## MEASURES FOR PREVENTING OTA APPEARANCE IN WINE AND POSSIBILITES OF ITS REMOVAL

If measures for prevention of OTA appearance, or its appearance in wine, are taken into consideration, they are primarily related to the production of grapes and are carried out all the way from planting of vineyard to harvest. As for wine production, one should know which operations in primary processing can contribute to wine contamination with this mycotoxin, as well as which treatments can reduce its content in wine.

When more important factors significant for OTA occurrence on grapes are considered, it is necessary to identify the types and species of mould existing in particular wineyard region. Moreover, information on microclimate and technology of vine cultivation are also very important. Vine growers should be informed about the risk of appearance of moulds and mycotoxins on grapes, and measures of prevention in vinegrowing. Apart from avoiding the terrain with higher relative humidity for planting vineyard, a direct contact of grape clusters with soil should also be avoided, and a good control of pests and vine diseases provided. Great help in prevention of OTA can be a choice of particular clones within some cultivars which are more adaptable under given ecological conditions and less susceptible to mould development. Apart from suitable vine protection programme it is also necessary to pay attention to leaf substance — grapes relation which is closely related to the application of nitrogen fertilizers, since they can stimulate excessive growth of vine. During ripening of grapes, works in vineyard should be avoided or brought to a minimum, in order to prevent moulds from the soil to reach the grapes. In the case of irrigation, it should be done evenly, in order to avoid the breaking of berries and mould development. Marc, which is a secondary product of wine production from grapes, should not be used for fertilizing vineyard if it is contaminated with OTA producing moulds. Defoliation in grape zone during the ripening phase enables better ventilation which reduces the risk of mould appearance. Necessary measures should be taken to prevent grape berry damage by insects (grape moth, wasps), various diseases and pests. If necessary, the registred programmes of protection against mould should be applied by using corresponding management in order to avoid the occurrence of resistence in mould.

If grapes are moderately contaminated with toxicogene moulds, they should be removed before or during harvest. The existence of inspection line is useful in winery where the grapes are placed upon reception, and damaged and mouldy bunches removed. Transportation of grapes to winery, after the harvest, should be carried out in the shortest time possible. Vessels (containers) used for transportation of grapes should be properly cleaned after each use.

Grapes significantly contaminated with mould cannot be used for production of rectified concentrated must (RTK) or wine for human consumption, but only for wine used for distillation.

In case of OTA contaminated grapes, certain operations and processes in technological procedure of grape processing are somewhat modified, or completely eliminated. So, for example, thermic treatment of marc and its prolonged maceration should be avoided, and straining of the same carried out at smaller pressures with compulsory avoiding of strainers with continuous work. In case of OTA contaminated red grapes, it is necessary to appraise possibility of producing rose wine. Application of pectolytic enzymes should be avoided as well, since maceration action increases must yield, spreads extraction of phenol compounds from skins, but also increases OTA content in wine. In production of white wines, purification of must by filtering, centrifuging or flotation is recommended. In the cases where there is a risk of OTA contamination, measuring of its content in must is recommended, as well as the treatment with the lowest effective doses for its removal without lossing aromatic substances and phenol compounds. Alcohol fermentation should last as long as possible using yeast with good adsorption characteristics in regard to OTA.

Apart from wine yeast, partial reduction of OTA can be also achieved by means of suitable preparation of lactic acid bacteria which displays good adsorption with regard to OTA. After alcohol fermentation, aging of wine on yeast reduces OTA level, but it is necessary to have in mind the possible influence of this on wine quality. Organic and inorganic clarification substances have different adsorption with regard to OTA. Some preparations based on cellulose, as well as on silica gel combined with gelatin, are capable of partial reduction of OTA content in wine, but active oenology coal, by itself or in combination with other means, displayed the best efficiency in that respect.

Abrunhosa et al. (2005) found that OTA content diminishes during alcohol fermentation, and if malolactic fermentation is carried out as well, OTA content in wine is about 32% in relation to the quantity found in grapes. Considerable quantity of OTA, 50% ± 10,3, was found in lees after completion of alcohol fermentation. Addition of OTA values in wine and lees represented approximately total OTA quantity found in grapes. After separating from lees, OTA quantity was only about 11% of its value in grapes, while level of OTA in lees was 80%. In this research, malolactic fermentation caused reduction of OTA to about 3% of the quantity found in grapes. Wine settling agents can make a contribution to further reduction of OTA content, but application of effective doses is often limited by their adverse influence on wine colour and aroma. According to the above stated authors, some of the commercial enzymes are also able to reduce OTA level in wine having hydrolytic influence on it, which requires confirmation in further research.

Silva et al. (2007) have carried out extensive research on possibilities of OTA reduction in wine by means of various agents for treating wine (active coal, active coal combined with K-caseinate or silica gel, PVPP, cellulose, gluten and peas protein) and yeast, as well as preparations made out of yeast cell walls and hulls. Application of agent called ATOS (active coal and K-caseinate) in quantity 0,2 g/l reduced OTA content by 55%, while treatment with 0,5 g/l of this agent reduced OTA content by as much as 90% in relation to the initial content in wine. However, using up to 0,2 g/l of ATOS does not have more significant influence on colour intensity in red wines, while at higher doses wine gets noticeably colourless, which depends on type of active coal as well. Out of seven used active coal preparations (10 and 50 g/hl) two displayed very weak ability of OTA absorption, while with the others, using 50 g/hl for 24 hours, reduction of OTA ranged even up to 90%. As for using PVPP, cellulose, gluten and peas protein, the experiments show that PVPP (40 and 80 g/hl) and cellulose (10 and 30 g/hl) reduce OTA content by approximately the same per cent (from 28—42%) when remaining in contact with wine for 48 hours. Gluten and peas protein showed approximately the same effect, a reduction of OTA content in wine by 39 — 45% after 24 hour application. As for yeast, dried active, inactivated, cell walls and yeast cell hulls, their effectiveness in reducing OTA in wine differs. In all stated treatments, contact of preparation and wine lasted for 8 days, and doses recommended by the producer have been applied. Preparation based on yeast cell walls was not used in the quantity of 100 g/hl and it did not give satisfactory results, while preparation based on yeast cell hulls reduced OTA content in wine by 40%. Inactivated yeast displayed poor activity in relation to OTA in wine, being applied in quantities of 20 and 40 g/hl it reduced OTA content by 25,8 and 26,3%. Preparation microsorb, based on yeast *Sacch. cerevisiae*, applied in quantity of 25 g/hl with 60 hour contact, reduced OTA by 77%, quantity of 50 g/hl reduced it by 81%, while quantity of 100 g/hl achieved OTA reduction by as much as 90%, but the intensity of wine colour was also reduced. Very strong point of this preparation is that it is highly effective in a very short period of time (60 hours).

The highest reduction of OTA content by yeasts and preparations based on their by — products has been achieved when contact with wine lasted for eight days at 20°C. Reduction ranged from 40—50% in cases when 100 g/hl of yeast cell walls were used, 40 g/hl yeast cell hulls, 200 g/hl active dried yeast and 10 g/l yeast cream were applied.

## CONCLUSION

During processing of grapes and vinification, a considerable reduction of OTA occurs, and purification of must and fermentation processes were the most effective in that respect. Also, some wine clarification agents can be of great help in that sense, but they should be applied exclusively in concentrations that do not violate sensory characteristics of wine.

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## МИКОТОКСИНИ У ВИНУ СА ПОСЕБНИМ ОСВРТОМ НА ОХРАТОКСИН А

Слободан М. Јовић, Александар В. Петровић, Небојша Р. Марковић

Пољопривредни факултет, Немањина 6, 11080 Земун, Србија

Резиме

Квалитет вина је комплексан, вишеслојни појам састављен од већег броја чинилаца попут сензорских карактеристика, хемијског састава, законодавно-правног, тржишно-потрошачког, при чему хигијенско-токсиколошки има посебан значај с обзиром на све израженије захтеве за здравствено безбедном храном. У раду су приказани резултати досадашњих истраживања микотоксина у вину (афлатоксини, трихотецен, патулин), при чему је посебна пажња посвећена најчешће присутном охратоксину А на грожђу, у шири и вину и утицај појединих технолошких операција и процеса у току производње вина на његов садржај. С обзиром на његову високу токсичност Европска Унија ЕГ регулативом 123/2005. свела је највише дозвољену количину охратоксина А на 2 μg/l.