# METHODS FOR ASSESSING THE MYCOTOXINS POTENTIAL IN OENOLOGY INDUSTRY

## METODE DE EVALUARE A POTENȚIALULUI ÎN MICOTOXINE DIN INDUSTRIA OENOLOGICĂ

***NISTOR Alina-Mihaela[1], COTEA V.V.[1], NICULAUA M.[2], LUCHIAN Camelia Elena[1], COTAN Şt.D.[1]***
e-mail: alinamihaela.nistor20@gmail.com

***Abstract:*** *Recent studies have confirmed that mycotoxins such as aflatoxins are present in foods and are responsible for most cases of liver cancer and modification of human DNA. These forms on foods where conditions of handling, transportation and storage promotemould growth, such as high temperature and humidity. There are four types of aflatoxins in food isolates (B1, B2, G1, G2) with B1 the most toxic. The study aims to assess methods of analysis for these important substances in human health and quality of wine. The maximum authorized concentration of this class of mycotoxins is 2 ppb. The determination of aflatoxins in the wine can be achieved by high performance liquid chromatography, thin layer chromatography, gas chromatography and ELISA. The purpose of this work is to stimulate risk awareness to the improper selection of harvest quality and the need to invest in the product safety.*
**Key words:** grapes, mycotoxins, toxicity

***Rezumat:*** *Studiile recente au confirmat faptul că micotoxine precum aflatoxinele sunt prezente în alimente şi sunt responsabile de cele mai multe cazuri de apariție a cancerului hepatic precum şi denaturarea ADN-ului la om. Acestea se formează pe alimente în cazurile în care condițiile de manevrare, transportare şi depozitare favorizează apariția mucegaiului, precum umiditate şi temperatură ridicată. Există patru tipuri de aflatoxine izolate din alimente (B1, B2, G1, G2), iar B1 are toxicitatea cea mai mare. Studiul îşi propune evaluarea metodelor de analiză pentru aceste substanțe importante pentru sănătatea umană şi calitatea vinului. Concentrația maximă autorizată ale acestei clase de micotoxine este 2 ppb. Determinarea aflatoxinelor din vin se poate realiza cu cromatografia de lichide de înaltă performanță, cromatografia în strat subțire, tehnica ELISA şi cromatografia de gaze. Scopul lucrării este conştientizarea riscului pe care îl reprezintă selecția necorespunzătoare a recoltei şi necesitatea investițiilor în cea ce priveşte calitatea produsului.*
**Cuvinte cheie:** struguri, micotoxine, toxicitate

## INTRODUCTION

The grape is one of the most popular fruit in the world, intended mostly for wine making, namely 71% of its production, 27% is consumed fresh and 2% is converted into dried fruits (raisins). Regarding the quality of the wine we

[1]University of Agricultural Sciences and Veterinary Medicine Iasi, Romania
[2]Reserch Centre for Oenology, Romanian Academy Iaşi branch, Romania

recognise two aspects: first the external quality that refers to the sensory characteristics and secondly that domestic quality is given by the chemical composition, hygiene factors related to wine toxicology.

In the last twenty years we have studied a lot of wine toxicity makers, with particular interest in mycotoxins. For these reason government agency tightened harvesting and processing conditions for winemakers and wanted to develop new techniques for identifying, quantifying and reducing mycotoxins in wine (Somma *et al.,* 2012).

Mycotoxins are metabolic by-products formed by moulds (Somma *et al.,* 2012), hence their name (Online Etymology Dictionary). Estimates worldwide show that at least a quarter of crops are affected by mould, which implies the risk of mycotoxin poisoning and possible economic losses (Cazzaniga *et al*., 2001; SommaS. et al., 2012). There are several types of fungi that lead to mycotoxins, but the most common include *Aspergillus*, *Alternaria*, *Penicillium* (Valero *et al.* 2005; Neacşu, 2012). Diseases caused by these toxins are called generally "*mycotoxicoses*" (Bennett and Klich, 2003). The most important classes of mycotoxins due to toxicity and their influence on human health and their correspondence in oenology are: Aflatoxins, Ochratoxins, Citrinin, Fumonisins, Patulin, Trichothecenes and Zearalenone.

Aflatoxins are produced by the fungus *Aspergillus flavus*, from which they were given the name (A -fla -toxin) (Sargeant *et al.,* 1963). They were first discovered in 1960 when an aviary pandemic occurred to 100,000 young turkeys and 20,000 ducks, pheasants and partridges. They all were strongly infected with *Aspergillus flavus* that developed as fodder came into contact with birds. Samples were taken from the feed and were analysed by Thin-Layer Chromatography (TLC). Thus they were observed on pads by UV fluorescence where two components one blue and one colour green, which were named"Aflatoxin B" and "Aflatoxin G"(Jacobsen *et al.,* 1993; Rustom, 1997; Devero, 1999; Sargeant *et al.,* 1963). It was later found about twenty compounds, but the most common are aflatoxins B1, B2, G1, G2 and two B1 metabolites, M1 and M2 (Agag, 2004). Aflatoxin B1 is 60-80% of the total aflatoxins (FDA, 1979) and is the most toxic natural carcinogen (Jones *et al*., 1994; Jović *et al.,* 2009). "International Agency for Cancer on Research" framed aflatoxins in group I, i.e.carcinogenic compounds for the human body (IARC, 2016), targeting mainlythe liver (Agag, 2004).

Contamination of wine and thus grapes can occur in two ways. First when developing culture is affected physically (by insects, birds, mammals, etc.) or mechanical (by hail, storm) and subsequently subjected to stress caused by heat and drought conditions (Cotty and Lee, 1990; Dowd, 1998). The optimum temperatures for the development of *A. flavus* species are between 25-32 $^{0}$C (Lillehoj, 1983). Thus, were created favourable conditions develop mildew on grapes after due improper sorting before processing, are a major risk factor for wine quality.Secondly, the healthy grapes which after once harvested are not deposited, transported or handled appropriately and due higher temperatures and

humidity, the grapes aremould growth predisposed that causing toxins (Cotty, 1991; Russell *et al*., 1976). In these conditions it is necessary that the time from harvest to processing to be minimized.

Ochratoxins were first discovered in 1965, as produced by *Aspergillus ochraceus* (Jović et al., 2009). Currently known several types but the most popular from the point of view of toxicity is "Ochratoxin A". It has been classified by the "International Agency for Cancer on Research" in group 2B carcinogen (substance possibly carcinogenic to humans) (IARC, 2016). According to estimates *Codex Alimentarius* approximate 15% of the total ochratoxin ingested by humans comes from wine and especially from wine red which is the second source of ochratoxin, according to statistics of FAO and *Codex Alimentarius* (Jović *et al.,* 2009; *Codex Alimentarius* Commission). Ochratoxins have hepato-nephrotoxic actions but is not a very high risk to human health (Somma, 2012). They were detected in grapes before harvest (Aziz and Moussa, 2002). Maximum permitted quantity of aflatoxins and ochratoxins in grape juice and wine, according to the regulations E.U. is 2ppb (Vicam, 2016).

**Fumonisins** was first time studied in 1988 (Bezuidenhout, 1988; Gelderblom, 1988) as the product of a number of species such as *Fusarium*, particularly *Fusarium verticillioides* (Rheeder, 2002). When weather conditions are unfavourable and the culture is attacked by insects and pests, on the stem grow rot and seedlings are affected by downy mildew (Nelson, 1993). IARC classified the toxin in group 2B carcinogen.

**Patulin** was first isolated in 1940 from *Penicillium patulum* and used for its antibacterial and antiviral properties, but later was found to contain toxicity to both plants and animals. In 1960 it was classified as mycotoxin and the W.H.O. has established a tolerable upper limit of 0.4 mg/kg bw/day. The most common form in which we find in fruits is "blue mould" caused by *Penicillium expansum* (Bennett and Klich, 2003).

**Trichothecenes** are produced by several types of fungi such as *Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma*, *Trichothecium*. Trichothecenes intoxications causing gastrointestinal bleeding, vomiting, damage to the small intestine and direct contact with mold can cause dermatitis. Trichothecenescan found in grapes and wine but in very rare cases (Jović *et al.,* 2009; Bennett and Klich, 2003; Somma, 2012).

## MATERIAL AND METHOD

First it is necessary to realize a more accurate sampling. On this line we must have in mind that grapes are contaminated with mycotoxins heterogeneous and then we should use correct sampling techniques to achieve as representative analysis.

To prepare samples for analysis we can take into account several extraction techniques. The clean-up for the samples is done generally by Solid Phase Extraction (SPE), but can try more advanced techniques, such as Solid Phase MicroExtraction (SPME) or we can provide better recovery and higher sensitivity withStir Bar Sorption Extraction (SBSE) (SBSE, Gerestel). In order to remove interference the desired compounds are concentrated in a solvent of choice to improve selectivity (Rahmani, *et al.*, 2008).

Actual analysis of mycotoxins requires the separation of interest compounds and their detection. The most effective are chromatographic techniques. Thin layer chromatography (TLC) is the first method by which identified aflatoxins and remains one of the most reliablefor common laboratories. It was adopted as official method and may to identify and quantify concentrations below 1 ng/g. Currently is developed a technique more advanced by using smaller particles layers for better selectivityHigh Performance Thin Layer Chromatography (HPTLC). It has a maximum thicknessof layer of 100 μmand particle size of stationary phase is between 2-10 μm, contributing to an advanced separation in a much shorter time. This makes the method to be comparable in terms of effectiveness with HPLC and ELISA methods. Another method developed for aflatoxin analysis is OverPressured-Layer Chromatography (OPLC) which unlike HPLC require less mobile phase and compared to TLC provides better resolution and more compact spots (Rahmani *et al.,* 2008).

High Performance Liquid Chromatography (HPLC) is able to detect all types of mycotoxins and is the most commonly used method for its small amount of volatile solvent consumption. For different types of detectors:UV hasa limit of detection 10 ng/g, DAD (PDA) has a limit of detection at 0.03 ng/g, and fluorescence detector (FLD)technique is the best spectrometric method. Citrinin could be detected by this method with a detection limit of 10 ng/g. Coupling Liquid Chromatography with Mass Spectrometry (LC-MS) results a more sensitive and selective. In this case, new techniques have been developed for better identification and quantification. For example, Trichothecenes were determined using Atmospheric Pressure Chemical Ionization (APCI), aflatoxins and ochratoxins byElectroSprayIonizationtechnique (ESI).A great sensitivity for low levels of ochratoxin concentration in the wine was obtained using clean-up with SPE C18 combined LC-ESI-MS/MS. For fumonisins LC-MS has 40 pg/Kg and most sensitive and accurate LC-MS/MS yielding a detection limit at 12 pg/Kg. To determine mycotoxins the best analysersare ion trap, time-of flight andtriple quadrupole mass analysers (Rahmani *et al.,* 2008).

Wine mycotoxins canalso be determined by Gas Chromatographycoupled with mass spectrometry (GC-MS) or a flame ionisation detector (GC-FID) to obtain the better results. By this technique ochratoxins have a quantification limit of 2 μg/L (Rahmani *et al.,* 2008).

Also common methods used in the analysis of mycotoxins, particularly aflatoxins are the ELISA tests (Enzyme linked immunosorbent assay). These clinical methods have good recoveries between 91% and 104%with a limit of detection at 0.01 μg/L (Rahmani *et al.,* 2008).

## RESULTS AND DISCUSSIONS

Whichever technique of analysingmould toxins is used, it must be specific enough, fast and it must help us get low detection and quantification limits. One the right concentration of the mycotoxins present in food are identified the manufacturers must design a strategy to reduce the level of contamination.

The safety measures for obtaining a safe wine must be applied starting from the planting of the vine until harvest time. In this case it must be taken into account the climate type, avoiding soils with high humidity, protecting the crops from pests and potential diseases (Jović *et al.,* 2009). The selection of the grapes during harvest and before processing must be done in a way such that the risk of contamination is reduced as much as possible. This can be done easily with the new grape sorting technologies. One choice would be using the machine that uses

a blowing mechanism to select the grape by its specific weight which would help remove the dry or mould damaged grape (e.g. Delta RFlow). Another choice would be optical selection using the machine which selects the grape by its colour, being able to remove mould damaged grapes, whose appearance is changed (e.g. Delta Vistalys by BUCHER).

Once the selection was done qualitatively the next step is to store, transport and process the grapes in good hygienic conditions keeping them away from humidity and high temperatures. In case the product is contaminated in this step, there are a few treatments that can still be applied to the wine to lower the mycotoxin concentration: a yeast with good absorbing properties could be used during the alcoholic fermentation process; clearing fabrics that are able to absorb mycotoxins could be used, such as those based on silicagel or cellulose, but all of these also have disadvantages and can badly influence the sensorial properties (Jović *et al.,* 2009).

## CONCLUSIONS

Currently the requirements regarding wine quality and human health need investment and involvement both from the producers, with modern harvesting and processing technologies, and from the analysts, with faster and more sensitive mycotoxin determination and quantification methods.

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