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ORIGINAL RESEARCH PAPER

QUANTIFICATION OF OCHRATOXIN A IN MOLDAVIAN WINES

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Abstract: The level of the carcinogenic mycotoxin ochratoxin A (OTA) in wines produced in Moldova, including bottled and raw material wines, was analyzed by enzyme-linked immunosorbent assay (ELISA) and high performance liquid chromatography (HPLC) analysis with fluorescence detection after immunoaffinity column clean-up. The study was conducted on wine samples from vintage 2006 - 2016. Our results confirm previously published reports that the levels of OTA are considerably higher in red wines than those in white ones. It was found that OTA levels of analyzed samples differ significantly depending on the harvest year of the wine. Thus, 48 % of studied wine samples from vintage 2006 were contaminated with OTA, including 10 % samples with concentration of OTA higher than 2 $\mu g \cdot L^{-1}$. In contrast, levels of OTA detected in samples from 2016, haven't exceed 0.05 $\mu g \cdot L^{-1}$ (quantification limit of HPLC method).

Keywords: *ELISA, HPLC, mycotoxin, ochratoxin A, wine*

INTRODUCTION

Mycotoxins are secondary metabolites of microfungi that are known to cause sickness or death in humans or animals. Ochratoxin A (OTA) is naturally occurring mycotoxin produced by fungi *Aspergillus ochraceus*, *Carbonarius* and *Penicillium verrucosum*. *Aspergillus ochraceus* is typically associated with coffee, grapes and spices [1]. OTA has a strong liver and kidney toxicity; in addition it exhibits teratogenic, mutagenic and carcinogenic properties, and thus poses a serious health problem for humans and animals [2 – 5]. Since OTA is relatively stable like other mycotoxins within the range of conventional food processing temperatures, and partially degraded under fermenting process, it can be detected in various manufactured food products [6]. This study aims to determine the level of OTA contamination and monitor its concentration during winemaking process for wines, produced in Moldova, including bottled and raw material wine: red, white, dry and sweet wines. Concentration of OTA was determined with ELISA and HPLC methods of analysis. Enzyme-linked immunosorbent assay (ELISA) has become a popular and useful screening tool due to the availability of polyclonal and monoclonal antibodies against OTA [7, 8]. ELISA was used as reliable and fast method for determination of OTA. ELISA test kits are favored because they require low sample volume and quicker sample clean-up than the conventional methods such as HPLC. ELISA is a technique based on the reaction of antigen–antibody, but antibodies sometimes show cross-reactivity to compounds similar to mycotoxins [9].
This research includes investigation of possible treatments on wine samples contaminated with OTA in order to prevent the ingress of OTA into the final wine product.

MATERIALS AND METHODS

Chemical and reagents

For study were used such materials, as: OTA (> 98 %; Sigma-Aldrich, Germany); acetonitrile (MERCK, Germany) and methanol (Sigma-Aldrich, USA) were HPLC grade. Sodium chloride, sodium hydroxide, disodium hydrogen phosphate, potassium dihydrogen phosphate, glacial acetic acid and potassium chloride were of analytical grade (purity min 99 %). The used water was obtained from a Milli-Q purification system (Milli-Q® IQ 7000, MERCK, Germany).

Wine samples

This study included 956 samples, including raw material wine, dry wines, semi-dry, semi-sweet and sweet wines. Wine samples produced from different grape varieties (414 samples of white wine: Sauvignon, Chardonnay, Muscat; and 542 of red and rose wine samples: Cabernet, Merlot, Pinot Noir, etc.) were made by Moldavian wineries and analyzed for presence of OTA during certification process in the Laboratory of National Center for Quality Testing of Alcoholic Beverages. Collected data cover vintages from 2006 to 2016.

Methods of analysis

ELISA assays

ELISA principle bases on the next interaction: an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme. Free OTA in sample solutions and the immobilized antigen compete for Ochratoxin enzyme conjugate. Addition of the substrate solution (horseradish peroxidase (HRP)) leads to the formation of a color signal, that inversely proportional to the concentration of OTA present in the sample. Scheme of ELISA procedure is shown in Figure 1.

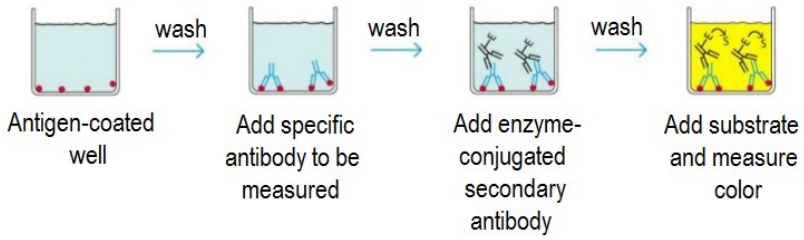

Figure 1. *Scheme of ELISA procedure*

RIDASCREEN©FAST Ochratoxin A (R-Biopharm, Scotland) were the ELISA test kits used in the present study.

HPLC method

For HPLC analysis, an aliquot of 10.0 mL of wine or must sample was taken, the *p*H was adjusted to 7.0 - 7.5 with 2M NaOH, then diluted with 10.0 mL of a phosphate buffer (*p*H 7.4). The obtained solution was applied onto an immunoaffinity column (IAC, OchraTest columns, Vicam). The column was washed with 20.0 mL phosphate buffer at a flow of 1 or 2 drops per second. Column was dried by blowing air through it. OTA was eluted from the column using 2.0 mL of methanol acidified with acetic acid (98:2 v/v) at the rate of one drop per second. The eluate was collected and diluted with 3.0 mL of mobile phase for HPLC analysis. OTA was identified by retention time and quantified by comparison with peak areas of the standards. The standard curve was linear ($r^2 = 0.9994$), with the detection limit 0.05 $\mu g \cdot L^{-1}$.
Chromatographic conditions are shown in Table 1.

Table 1. *Chromatographic conditions*

Chromatographic column	ES 160/4.6, Nucleosil 100-5, C_{18} HD, 5µm, 150 x 4.6 mm (Macherey-Nagel, Germany)
Mobile phase	Acetonitrile : water : acetic acid = 99 : 99 : 2
Flow	1.0 $mL \cdot min^{-1}$
Fluorescence detector	Excitation wavelength – 333 nm Emission wavelength – 420 nm
Volume of injection	100 µL
Oven temperature	40 °C

High performance liquid chromatography (HPLC) analysis was performed using a Shimadzu LC-20AD (by Shimadzu USA Manufacturing Inc.) chromatography system.

RESULTS AND DISCUSSIONS

In 2006, the percentage of contaminated samples was near 48 %, reaching in 2007 up to 55 %. The number of contaminated samples reduced to 0 % in 2016 – number of wine samples with concentration of OTA less than 0.05 $\mu g \cdot L^{-1}$ (LQ of HPLC method of analysis, 0.1 $\mu g \cdot L^{-1}$ – LQ of ELISA). Only in the case of 1.4 % the total number of studied samples, the determined amount of OTA was higher than the maximum allowable concentration of 2.0 $\mu g \cdot L^{-1}$. This percentage consists almost exclusively of raw wine material, for which further processing could reduce OTA content during alcoholic fermentation, bottle-aging and other stages of the winemaking process [10 – 12]. Results of research are shown in Table 2.

Table 2. *Results of analysis of wine samples from 2006 to 2016 years*

Vintage	Wine type / OTA concentration [$\mu g \cdot L^{-1}$]								
	Raw wine material			Dry wines			Semi-dry, semi-sweet, sweet wines		
	< 0.1	0.1 - 2.0	> 2.0	< 0.1	0.1 - 2.0	> 2.0	< 0.1	0.1 - 2.0	> 2.0
2006	13	11	3	11	4	2	3	5	-
2007	12	17	8	8	12	-	27	21	-
2008	15	12	1	7	12	-	23	23	-
2009	25	14	-	29	8	-	32	11	-
2010	32	7	-	30	5	-	25	3	-
2011	37	5	-	33	1	-	14	1	-
2012	44	-	-	37	-	-	22	-	-
2013	52	-	-	25	-	-	17	-	-
2014	53	-	-	11	-	-	14	-	-
2015	37	-	-	17	-	-	20	-	-
2016	25	-	-	13	-	-	7	-	-

The OTA content of wines, indicating color, are shown in Table 3. Noticeably, OTA was detected more often in red wines than in white ones. In addition, the OTA concentration in the red wines was remarkably higher than in the white wine samples, according to the literature [13]. Obviously, that took place because the maceration of pomace has a significant effect on the increase of OTA content in red wines [14].

Table 3. *OTA content in wines depending of color*

Type of wine	OTA concentration [$\mu g \cdot L^{-1}$]			
	< 0.3	0.3 - 0.9	1.0 - 2.0	Average
Red wine	29	38	33	0.86
White wine	48	16	8	0.27

Changes of OTA content during the bottle-aging process are not well investigated. Several samples of each type (5 red and 5 white wine samples) were analyzed. The average OTA content decrease was more than 40 % of the initial content. Probably, OTA was adsorbed on the sedimentation substances formed during the aging process.
No treatment could reduce OTA content to zero. But some stages of the winemaking process, such as: alcoholic and malo-lactic fermentation [15], using of fining agents:

activated carbon, silica gel, potassium caseinate, egg albumin, gelatin [16, 17], yeast treatment [18 – 20], lead to the reduction of OTA content in the wine.
Activated carbon showed the best results and the highest adsorption capacity of OTA, but its' utilization leads to damage to the nature of wine, reduce optical density on the wavelength of 280 nm, color intensity due to adsorption of polyphenols and anthocyans. Thus, wine treatment with activated carbon needs to be strongly controlled, especially for red and rose wines.
Contaminated samples were subdivided by their origin in two groups, produced from different grape varieties: *Vitis vinifera* (Merlot, Muscat Blank, Cabernet Sauvignon) – 152 samples; and *Vitis labrusca* (Lidia, Isabella – *Vitis vinifera, Vitis labrusca* hybrid grapes) – 38 samples. The results of our analysis showed that the wines produced from *V. vinifera* had the lower content of OTA than other wines. Such results require further investigations.

CONCLUSIONS

This study of musts and further stage of winemaking products explains which stages can reduce OTA concentration in wine. Wine fining and filtration partially removes OTA's from wine. Activated carbon showed the most effective results, but such treatment is unsuitable for red and rose wines. In addition, some yeasts strains allow reducing OTA concentration. Choosing the right yeasts can help to minimize OTA content when coupled with controlled maceration. In that prolonged maceration OTA content is decreased by extraction from contaminated pomace.
Strong control of factors affecting the presence of OTA in musts, such as: grape cultivation, presence of spoilage microorganisms, conditions of storage of the harvested grapes, and further stages of winemaking – only coupled together, these actions can be enough effective in production of wines free from OTA. Winemakers in Moldova have significantly decreased the concentration of OTA in their wines by reducing the sources of contamination.

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