Quantitative determination of ochratoxin A in wine after the clarification and filtration

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Summary

Through the use of the analytical method known as HPLC-FD and previously used the method for extraction of ochratoxin A by immunoaffinity columns, we have analyzed the possible effect of the clarification and filtration process on 54 samples of 2013 newly fermented wine, which have finished alcoholic fermentation process and racking, without knowing in advance whether there and how is the amount of OTA in wine. The racking of the wine before clarification and filtration followed by adequate clarification and filtration process during winemaking seems to be crucial in the reduction or complete elimination of the analyzed mycotoxin (OTA).The results of all analyzed samples have been below the limit allowed by the EU for ochratoxin A i.e. 2 ng/ml, and as such does not represent a risk to human health.

Keywords: wine, ochratoxin A, HPLC-FD, immunoaffinity column, mycotoxin

Introduction

Ochratoxin A, N-[(3R)-(5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl) carbonyl]-L-phenylalanine (Fig. 1) is a mycotoxin produced by certain species of *Aspergillus* and *Penicillium* filamentous fungi. The *Penicillium* species that is associated with ochratoxin A production, *Penicillium verrucosum*, is a common storage species and is the source of ochratoxin A in crops in the cool temperate regions such as Canada, eastern and north western Europe and parts of South America. It grows only at temperatures below 30 °C and at a lower water activity. *Penicillium* species may produce ochratoxin at temperatures as low as 5 °C (Risk Assessment Studies, http://www.cfs.gov.hk/).

Aspergillus species appears to be limited to conditions of high humidity and temperature growing in the tropical and subtropical climates and is the source of contamination for coffee and cocoa beans, spices, dried vine fruit, grape juice and wine. *Aspergillus ochraceus* is the best known species of ochratoxin-producing *Aspergillus*. It grows at moderate temperatures and at a high water activity and is a significant source of ochratoxin A in cereals. It infects coffee beans usually during sun-drying causing contamination in green coffee (Risk Assessment Studies, http://www.cfs.gov.hk/). *Aspergillus carbonarius* is highly resistant to sunlight and survives sun-drying because of its black spores and therefore grows at high temperatures. It is associated with maturing fruits and is the source of ochratoxin A in grapes, dried vine fruits, and wine and is also another source of ochratoxin A in coffee (Risk Assessment Studies, http://www.cfs.gov.hk/).

Aspergillus niger is another minor source of ochratoxin A production in infected coffee beans and dried vine fruits. The mycotoxin has been detected in various food stuffs such as dried fruits, coffee, maize, sorghum, wheat, pulses and wine (Marquardt and Frohlich, 1992; Steyn and Stander, 1999).

Ochratoxin A is a mycotoxin which is soluble in organic solvents, in aqueous solution of sodium bicarbonate and slightly soluble in water. The uniqueness of OTA is its high stability. It is shown that it has a pronounced resistance to acidity and high temperatures. Thus, when foods are contaminated, it is very difficult to remove completely. At normal cooking showed that OTA was only partially degraded (Muller, 1982). Moreover, this molecule can withstand steam sterilization three hours with high pressure 121 °C, and even at 250 °C its destruction is not complete.

Mycotoxins can cause serious health problems in animals and humans known as mycotoxicosis (Muller, 1983). Mycotoxins can be classified as hepatotoxins, nephrotoxins, neurotoxins, immunotoxins, teratogens, mutagens, carcinogens, allergens, and so forth. Some mycotoxins are specifically indicated or strongly suspected as the cause of severe human and animal diseases, such as Reye's disease, caused by aflatoxin B1 (AFB1), equine leukoencephalomalacia and porcine pulmonary edema, caused by fuminisin B1 (FB1), human alimentary toxic aleukia, caused by T2

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toxin, and Balkan endemic nephropathy, caused by ochratoxin A (Sorrenti et al., 2013). OTA is arguably a risk factor for Balkan endemic nephropathy (BEN). BEN is a chronic tubulointerstitial kidney disease that occurs in some areas of Bosnia and Herzegovina, Bulgaria, Croatia, Romania, Serbia, and Montenegro (Yordanova et al., 2010). Based on extensive research across the world has shown that the OTA is present not only in European countries but also in other continents of the globe. Scientific research has shown the histopathological changes to the kidneys and liver of rats (Aydin et al., 2013). OTA has been observed to be teratogenic in a number of animal models including rat, mouse, hamster, and chick, with reduced birth weight and craniofacial abnormalities being the most commonly observed malformations (O'Brien et al., 2005). OTA also has been found to have genotoxic and immunotoxic effect in Wistar male rats (Alvarez et al., 2004).OTA exposure has been associated with increased levels of oxidative DNA, lipid, and protein damage. Second, various biological processes known to be mobilized under oxidative stress were shown to be altered by OTA. These effects have been observed in both in vitro and in vivo test systems. In vivo, active doses were often within doses documented to induce renal tumors in rats (Marin-Kuan et al., 2011). International Agency for Cancer Research classifies OTA as potential carcinogenic substance for man (group 2B). Zimmerli and Dick (1995) were the first ones to report the existence of OTA in wine. The European Union Regulation (EC 123/2005) limit for OTA in wine is 2 ppb (µg/L).

The aim of the research

The aim of this research has been the analysis of the ochratoxin A (OTA) during a normal technological process of winemaking, respectively the possible movement of OTA amount during different stages of the technological process and the impact of these stages in the final wine ready for consumption.This research is of particular importance based on the fact that ochratoxin A (OTA) has multiple negative effects on the human body if its quantities in the wine exceeding limits permitted by the European Union (2 ppb).

Material and methods

Reagents and chemicals

OTA standard (Lot No: L13092B, 10.20 µg/ml) was obtained from LGC Standards (Wesel, Germany). All chemicals were of the analytical grade and solvents for mobile phase were of the HPLC grade. A stock solution of OTA was prepared in the mobile phase (100 ng OTA/ml). The working standards for HPLC analysis were prepared by adding known amounts of the diluted stock solution to the HPLC mobile phase to give final concentrations from 0.1 to 5.0 ng OTA/ml. The working standards were freshly prepared every day.

Sampling

Wine samples were taken from Agrokosova Holding Winery-Suhareka, Kosovo. The samples during technological process, after the racking stage, were treated with special clarification substances and special filter sheets, in order cleaning and filtering the wine. Substances which we have used for clarification process of the white wines have been: bentonite (50g/Hl) and PVPP-Polyvinylpolypyrolidone (10 g/Hl), while for red wines have been: bentonite (5 g/Hl), gelatine (10 g/Hl) and PVPP-Polyvinylpolypyrolidone (5 g/Hl). For wine filtration process we have used depth filter sheets-Europor-K range (K30 & K60, Begerow Company-Germany) and special wine filtration equipment (Della Toffola SPA, Italy). The samples for clarification process were taken from two of each wine tank (treated and untreated wine samples with clarification substances (Table 1)), and for filtering process were taken from three of each wine tank (untreated with filter sheets, filtered with K30 and filtered with K60 (Table 2)).

Extraction and clean – up

The method which we used for extraction and HPLC-FD analysis was the method which has been described by Visconti et al. (1999) for determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography. The wine was first diluted with so-called extraction solution containing 1% polyethylene glycol (PEG 8000) and 5% sodium hydrogencarbonate, filtered and applied to an Ochra Test immunoaffinity column, Vicam Inc (USA). The column was additional washing with a washing solution containing sodium chloride (2.5%) and sodium hydrogencarbonate (0.5%) followed by water and OTA was eluted with methanol.

HPLC conditions

The OTA in eluate was quantified by reversed-phase HPLC with fluorometric detection (excitation wavelength 333 nm, emission wavelength 460 nm), column nucleodur C18 (4.6 × 250 mm), size of particles 5µm (Machenrrey-Nagel, Germany),

software system ChromQuest 5.0, using acetonitrile-water-acetic acid (99:99:2) as mobile phase. The mobile phase was degassed first by sonication for 15 min in an ultrasonic bath. The flow rate was 1 ml/min and the injection of volume was 50 µl. Limit of detection (LOD) was 0. 05 ng/ml and limit of quantification (LOQ) was 0.1ng/ml. The retention time was 8 minute.

Table 1. OTA concentration in analyzed wine samples by HPLC-FD before (untreated) and after clarification (gelatine, bentonite, PVPP)

Nr. of wine tank	Variety	Clarification substances gr/Hl	OTA ng/ml
T1	Sauvignon Blanc	bentonite 50 gr/Hl, PVPP 10 gr/Hl	< LOD
T1	Sauvignon Blanc	Untreated	0.015
T3	Cabernet Sauvignon	gelatine 10 gr/Hl, bentonite 5 gr/Hl, PVPP 5 gr/Hl	N.D.
T3	Cabernet Sauvignon	Untreated	N.D.
T5	Pinot Noir	gelatine 10 gr/Hl, bentonite 5 gr/Hl, PVPP 5 gr/Hl	N.D.
T5	Pinot Noir	Untreated	N.D
T6	Franconia	gelatine 10 gr/Hl, bentonite 5 gr/Hl, PVPP 5 gr/Hl	< LOD
T6	Franconia	Untretated	< LOD
T14	Pinot Blanc	bentonite 50 gr/Hl, PVPP 10 gr/Hl	< LOD
T14	Pinot Blanc	Untreated	< LOD
T 104	Rhine Riesling	bentonite 50 gr/Hl, PVPP 10 gr/Hl	N.D.
T 104	Rhine Riesling	Untreated	N.D.
T 111	Gamay Noir	gelatine 10 gr/Hl, bentonite 5 gr/Hl, PVPP 5 gr/Hl	N.D.
T 111	Gamay Noir	Untreated	< LOD
T 112	Pinot Blanc	bentonite 50 gr/Hl, PVPP 10 gr/Hl	N.D.
T 112	Pinot Blanc	Untreated	N.D.
T 108	Franconia	gelatine 10 gr/Hl, bentonite 5 gr/Hl, PVPP 5 gr/Hl	N.D.
T 108	Franconia	Untreated	N.D.
T 2	Pinot Blanc	bentonite 50 gr/Hl, PVPP 10 gr/Hl	< LOD
T 2	Pinot Blanc	Untreated	< LOD
T 17	Gamay Noir	gelatine 10 gr/Hl, bentonite 5 gr/Hl, PVPP 5 gr/Hl	N.D.
T 17	Gamay Noir	Untreated	N.D.
T 18	Italian Riesling	bentonite 50 gr/Hl, PVPP 10 gr/Hl	N.D.
T 18	Italian Riesling	Untreated	N.D.
T 102	Italian Riesling	bentonite 50 gr/Hl, PVPP 10 gr/Hl	N.D.
T 102	Italian Riesling	Untreated	N.D.
T 107	Franconia	gelatine 10 gr/Hl, bentonite 5 gr/Hl, PVPP 5 gr/Hl	N.D.
T 107	Franconia	Untreated	< LOD
T 110	Franconia	gelatine 10 gr/Hl, bentonite 5 gr/Hl, PVPP 5 gr/Hl	< LOD
T 110	Franconia	Untreated	< LOQ

Note. LOD = limit of detection, LOQ = limit of quantification, N.D. = not detected, PVPP = polyvinylpolypyrrolidone, HPLC-FD = High Performance Liquid Chromatography with Fluorescence Detection, HL = 0.01 L

Table 2. OTA concentration in analyzed wine samples by HPLC-FD before (unfiltered) and after filtration (K30, K60)

Nr. of wine tank	Variety	Filtration	OTA ng/ml
T1	Sauvignon Blanc	Unfiltered	0.015
T1	Sauvignon Blanc	K 30	< LOD
T1	Sauvignon Blanc	K 60	N.D.
T14	Pinot Blanc	Unfiltered	< LOD
T14	Pinot Blanc	K 30	< LOD
T14	Pinot Blanc	K 60	N.D.
T17	Gamay Noir	Unfiltered	< LOD
T17	Gamay Noir	K30	< LOD
T17	Gamay Noir	K 60	N.D.
T107	Franconia	Unfiltered	0.008
T107	Franconia	K30	< LOD
T107	Franconia	K 60	N.D.
T104	Rhine Riesling	Unfiltered	< LOD
T104	Rhine Riesling	K30	N.D.
T104	Rhine Riesling	K 60	N.D.
T111	Gamay Noir	Unfiltered	< LOD
T111	Gamay Noir	K30	< LOD
T111	Gamay Noir	K60	N.D.
T108	Franconia	Unfiltered	< LOD
T108	Franconia	K30	< LOD
T108	Franconia	K60	N.D.
T 102	Italian Riesling	Unfiltered	< LOD
T 102	Italian Riesling	K30	N.D
T 102	Italian Riesling	K60	N.D

Note. LOD = limit of detection, LOQ = limit of quantification, N.D. = not detected, HPLC-FD = High Performance Liquid Chromatography with Fluorescence Detection, K30 & K60 = filter sheets, HL = 0.01 L

Results and discussion

It was determined that the amount of OTA in all analysed samples does not exceed the maximum level allowed by the European Union for this mycotoxin, which is 2ng/ml. From these results we can see that in almost all samples analyzed the amount of OTA is below the detection limit (LOD) or not detected (N.D.) at all. Samples for analysis were taken without knowing in advance whether or not OTA is present in wine samples and in order to see the potential effect in amount of ochratoxin A of clarification substances and filtration process during winemaking. From these results we can see also that the racking of the wine before clarification and filtration process during winemaking appears to be crucial in the reduction or complete elimination of the analyzed mycotoxin (OTA). For example, in the variety Sauvignon Blanc earlier analyzed immediately after alcoholic fermentation process (Durguti et.al, 2014), the amount of OTA it was 1.297 ng/ml, while a similar variety from the same tank after the racking and before the clarification and filtration has the amount of OTA 0.015 ng/ml. There are also other scientific works that indicated the fact for a reduction of OTA after the racking and after the treatment of the wine with different substances during winemaking. For example, the review from the University Do Minho (2010, Portugal) indicate a significant reduction of OTA during alcoholic fermentation, malolactic fermentation, after the racking and after the treatment of the wine with different technological substances (clarification substances). Another important research is carried out by Anabela et.al. (2007), which research shows also for a significant reduction of OTA during different stages of winemaking. During our research we have seen too that the proper clarification and filtration of the different varieties helps in the reduction or complete elimination of ochratoxin A during winemaking. Specifically, if we analyze the result of the variety Sauvignon Blanc before and after treatment with the clarification substances (Table 1) we can see a decrease in the amount of OTA that we have analyzed from 0.015 ng/ml (before clarification) to < LOD (after clarification). Similar results with complete elimination of OTA can be seen even after the filtration process. The amount of OTA in Sauvignon Blanc before filtration process it was 0.015 ng/ml, after the treatment with K30 filters it was < LOD and after the treatment with K60 filters we can

see the complete elimination of ochratoxin A (Table 2). From the obtained results we can see that in all analyzed samples after application of the K60 filters the quantities of OTA can't be detected.

Conclusions

During winemaking if the harvesting process, collection, transportation and processing of uninfected grape is done in normal conditions, then during different stages of production of the wine as are: racking, clarification, stabilization, filtering etc. the amount of mycotoxins including OTA will be reduced and finally we will have the wine with traces of OTA or the wine completely cleared by the presence of ochratoxin A. This research do not shows the risk of drinking the wine that we have analyzed in the future by consumers and as such do not represent a risk for human health. Based on these data, we recommend all wine cellars technologists, first of all to maintain the purity of the wine cellars and equipment at proper level and then strict application of all work protocols during winemaking.

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