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QUANTITATIVE DETERMINATION OF OCHRATOXIN A IN WINE

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Abstract

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 25 samples of 2013 newly fermented wine, which have just finished alcoholic fermentation process, 11 of which have been analyzed from different regions of Italy and 14 other samples from two regions of Kosovo. From the total number of samples, 24 samples were red wine and only one was white wine. The results of all analyzed samples have been below the limit allowed by the EU for ochratoxin A i.e. 2 ng/ml.

Key words: *wine, ochratoxin A, HPLC-FD, immunoaffinity column, myxotocin*

1. 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 spices 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 wich 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 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 Monte Negro (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

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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).

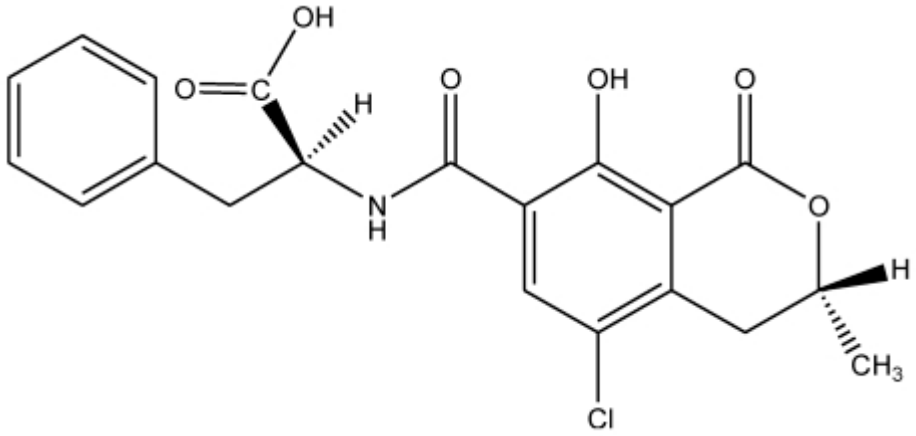


Fig.1. Chemical Structure of Ochratoxin A

2. MATERIAL AND METHODS

2.1. *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 0.05 to 5.0 ng OTA/ml. The working standards were freshly prepared every day.

2. 2. *Sampling*

Wine samples were taken from different regions of Italy and two regions of Kosovo. Samples were taken from several wine cellars which operate in the respective regions.25 wine samples were analyzed, 24 of which have been red wines samples and only one was white wine. The sample of white wine was taken for comparing with other red wine samples and the fact that white wine sample has been the product of extremely rotten and infected grape. All 25 analyzed samples have been 2013 newly fermented wine, which have just finished alcoholic fermentation process (Table 1).

2.3. *Extraction and clean – up*

The method wich we used for extraction and HPLC-FD analysis was the method wich 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. OTA was eluted with methanol and 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 (

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Machenrrey – Nagel, Germany), software system ChromQuest 5.0, using acetonitrile-water-acetic acid (99:99:2) as mobile phase.

Variety	Region	Country	Year
Refosco	Veneto	Italy	2013
Merlot	Calabria	Italy	2013
Pinot Noir	Veneto	Italy	2013
Magliocco Canino	Calabria	Italy	2013
Merlot	Friuli	Italy	2013
Sangiovese	Calabria	Italy	2013
Merlot	Veneto	Italy	2013
Merlot	Veneto	Italy	2013
Merlot	Veneto	Italy	2013
Refosco	Veneto	Italy	2013
Merlot	Veneto	Italy	2013
Cabernet Sauvignon	Rahovec	Kosovo	2013
Variety	**Region**	**Country**	**Year**
Sheshi i Zi	Rahovec	Kosovo	2013
Vranac	Rahovec	Kosovo	2013
Gamay Noir	Suhareka	Kosovo	2013
Gamay Noir	Suhareka	Kosovo	2013
Franconia	Suhareka	Kosovo	2013
Vranac	Rahovec	Kosovo	2013
Pinot Noir	Rahovec	Kosovo	2013
Gamay Noir	Rahovec	Kosovo	2013
Vranac	Rahovec	Kosovo	2013
Franconia	Suhareka	Kosovo	2013
Franconia	Suhareka	Kosovo	2013
Vranac	Rahovec	Kosovo	2013
Sauvignon Blanc	Suhareka	Kosovo	2013

Tab.1. (Distribution of wine samples) analyzed by HPLC-FD

2.4. *HPLC conditions*

The mobile phase consisting of acetonitrile-water-acetic acid (99:99:2) 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 11 minute. The excitation wavelength of the fluorescence detector was set at 336 nm, and the emission wavelength was set at 464 nm.

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3. 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 2 ng / ml. Improving the conditions of grape processing and wine production in the wine cellars of the countries of the European Union but also in Balkan countries including Kosovo, equipping these wine cellars with modern grape processing technology, may be one of the factors related with the presence of very small amounts of ochratoxin A in the samples that we have analyzed, the samples which present new wines which had just completed alcoholic fermentation process during vintage 2013. Is interesting to note that in the sample of white wine (Sauvignon Blanc) the amount of ochratoxin A was slightly greater (1.297 ng/ml) compared with other samples that normally does not happen, considering different ways of producing the red and white wine and of course the different likelihood for the presence of ochratoxin A based on these two different technologies (Fig 2). However as we mentioned above the white grape (Sauvignon Blanc) that we have taken for sampling has been extremely rotten and infected. Therefore special attention should be taken early in the process of harvesting the grapes and all other accompanying stages. 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 (Table 2).

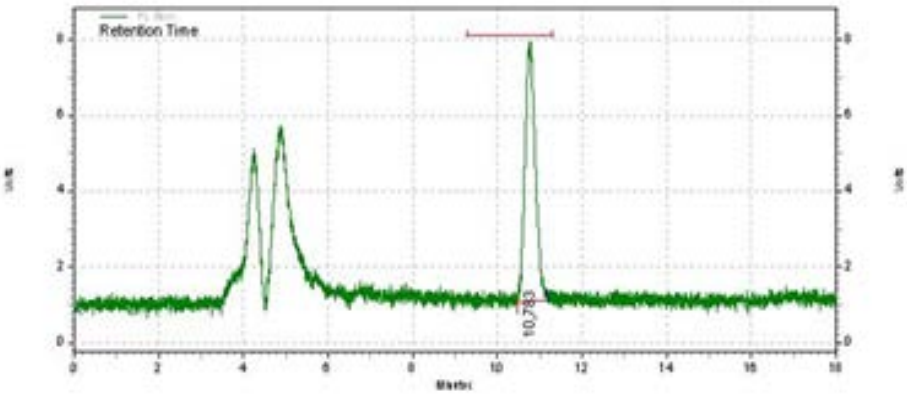


Fig.2. OTA concentration in Sauvignon Blanc sample

This research do not shows the risk of drinking the wine that we have analyzed in the future by consumers, because even though the wine samples are taken immediately after the alcoholic fermentation, the amounts of OTA were already in this stage in extremely small quantities, excluding sample 2 (Merlot) and 25 (Sauvignon Blanc) and as such do not represent a risk for human health.

Variety	Region	Country	Content OTA ng/ml ± SD
Refosco	Veneto	Italy	0.458 ± 0,048
Merlot	Calabria	Italy	1.431 ± 0,00
Pinot Noir	Veneto	Italy	0.805 ± 0,047
Magliocco Canino	Calabria	Italy	0.574 ± 0,032
Merlot	Friuli	Italy	0.847 ± 0,021
Sangiovese	Calabria	Italy	< LOD
Merlot	Veneto	Italy	< LOD

Merlot	Veneto	Italy	< LOD
Merlot	Veneto	Italy	< LOD
Refosco	Veneto	Italy	< LOD
Merlot	Veneto	Italy	< LOD
Cabernet Sauv.	Rahovec	Kosovo	< LOD
Sheshi i Zi	Rahovec	Kosovo	< LOD
Vranac	Rahovec	Kosovo	< LOD
Gamay Noir	Suhareka	Kosovo	< LOD
Gamay Noir	Suhareka	Kosovo	< LOD
Franconia	Suhareka	Kosovo	< LOD
Vranac	Rahovec	Kosovo	< LOD
Variety	**Region**	**Country**	**Content OTA ng/ml ± SD**
Pinot Noir	Rahovec	Kosovo	0.028 ± 0,001
Gamay Noir	Rahovec	Kosovo	0.129 ± 0,016
Vranac	Rahovec	Kosovo	0.286 ± 0,04
Franconia	Suhareka	Kosovo	0.348 ± 0,015
Franconia	Suhareka	Kosovo	0.119 ± 0,075
Vranac	Rahovec	Kosovo	0.111 ± 0,017
Sauvignon Blanc	Suhareka	Kosovo	1.297 ± 0,018

Tab.2. OTA concentration in analyzed wine samples by HPLC-FD

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