Hrana u zdravlju i bolesti, znanstveno-stručni časopis za nutricionizam i dijetetiku (2015) 4 (1) 11-15

QUANTITATIVE DETERMINATION OF OCHRATOXIN A IN BOTTLED WINE

Valon Durguti[1]*, Aneliya Georgieva[2], Angel Angelov [2], Zyri Bajrami[3]

[1]*College of Medical Sciences, Iliria - Rezonanca, Prishtina, Kosovo
[2]Department of Biotechnology - University of Food Technologies, Plovdiv, Bulgaria
[3]The Faculty of Natural Sciences – University of Tirana, Tirana, Albania

Abstract

In this study work we have determined the quantity of ochratoxin A (OTA) in 54 samples of bottled wine. The methodology which we used is the method known as High Performance Liquid Chromatography with Fluorescence Detection (HPLC). Before the HPLC analysis we have done the ochratoxin A extraction through the immunoaffinity clean-up procedure by high immunoaffinity columns. Samples that are analyzed have been taken in Kosovo and are wine samples produced and bottled in Republic of Kosovo and the wine samples produced, bottled and imported in Kosovo from other Balkan and European Union (EU) countries and beyond. The aim of this research has been the analysis of ochratoxin A in bottled wine for the first time in Kosovo and determination of the risk or not by consumption of the analyzed wines by consumers. 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 in the future do not pose a risk to human health.

Key words: 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-phe-nylalanine, is a mycotoxin produced by certain species of *Aspergillus* and *Penicillium* filamentous fungi. OTA contaminates cereals and cereal products, coffee, beans, pork meat and meat products, milk and milk products, eggs, wine, and beer all over the world (Flajs et al, 2009). Cereals and cereal products are the main sources of OTA intake, followed by wine, grape juice and coffee. The OTA levels in wine depend on different factors such as the climate, the date of harvesting and different wine-making procedures (Arbisu et al, 2010).The *Penicillium* species that is associated with ochratoxin A production, *Penicillium verrucosum* is an important ochratoxigenic species because it is the major producer of OTA in cereals such as wheat, barley, oats and rye, in temperate and cold climates (Cabañes et al, 2010). This species is the main source of OTA contamination in cereals associated to the porcine and avian nephropathy detected in temperate and cold countries such as Denmark, Sweden, Canada or the United States (Cabañes et al, 2010). At the moment, *P. verrucosum* and *P. nordicum* are the only OTA producing species accepted in the genus *Penicillium* (Cabañes et al, 2010). *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/). Aspergilli in vineyards varied depending on years and geographic areas: France, Greece and Israel were the areas with the highest incidence, followed by South Italy, Spain and Portugal (Oliveri et al, 2011). At normal cooking showed that OTA was only partially degraded (El Khoury et al, 2010). Moreover, this molecule can withstand steam sterilization three hours with high pressure 121 ° C, and even at 250 ° C its destruction is not complete (El Khoury et al, 2010).

*Corresponding author: loni_78@hotmail.com

The formation and occurrence of OTA in wines represents a serious economic problem in Europe because of it high share in world vineyard areas, which represent 75% of world-wide wine production.

Mycotoxins can cause serious health problems in animals and humans known as mycotoxicosis (Marquardt et al 1992).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. 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 purpose of this research has been the analysis for the first time of ochratoxin A in bottled wine produced in Kosovo and their comparison with imported bottled wines in Kosovo from the Balkan and European countries and beyond and defining the risk or not by consumption of these wines by consumers in the future. This research work is one of the stages in the framework of a scientific project that is implemented in Kosovo, involving the quantitative determination of ochratoxin A (OTA) from the beginning of the wine production to the final stage, which means the analysis of OTA in bottled wine. The wine samples taken for analysis are provided from the markets or wine cellars that operate in Kosovo and the selection is done spontaneously.

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 supermarkets and wine cellars which are operating in Kosovo. Bottle wine samples were taken for analysis and eventual determination of the presence and quantity of OTA in wine. From total of 54 analyzed samples of bottled wine, 36 samples were produced and packed in Kosovo, 3 in Albania, 2 in Montenegro, 3 in Macedonia, 3 in Bulgaria, 3 in Italy, 1 in France, 1 in Spain, 1 in Slovenia and 1 in Australia.40 analyzed bottled wine samples were red wine while 14 samples were white wine.Years of production and the percentage of alcohol in analyzed bottled wines were different.

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 ul-

trasonic 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.

Results and discussion

Table 1. OTA distribution in 36 analyzed bottled wine samples by HPLC-FD produced in Kosovo

Sample code	Name of the company	Variety	Country	Year of production	Volume of bottle / ml	Content of OTA ng / ml
K1	Murati	Vranac	Kosovo		187	< LOD
K2	Stone Castle	Vranac Premium	Kosovo	2011	187	< LOD
K3	Stone Castle	Chardonnay	Kosovo	2011	187	< LOD
K4	Biopak	Vranac	Kosovo	2010	187	< LOD
K5	Stone Castle	Cabernet Sauvignon	Kosovo	2011	187	< LOD
K6	EKO	Vranac	Kosovo		187	< LOD
K7	Bodrumi i Vjeter	Chardonnay	Kosovo	2012	187	< LOD
K8	Bodrumi i Vjeter	Vranç	Kosovo	2012	187	< LOD
K9	Rahoveci	Pinot Noir	Kosovo	2006	187	< LOD
K10	Iliria	Red Wine	Kosovo	2010	750	< LOD
K11	Theranda	White Wine	Kosovo	2009	750	< LOD
K12	Suhareka	White Wine	Kosovo	2012	750	< LOD
K13	Suhareka	Chardonnay	Kosovo	2012	750	< LOD
K14	Suhareka	Italian Rhiesling	Kosovo	2012	750	< LOD
K15	EKO	Cabernet Sauvignon	Kosovo		187	< LOD
K16	Iliria	Merlot	Kosovo	2008	750	< LOD
K17	Bodrumi i Vjeter	Merlot	Kosovo	2012	187	N.D.
K18	Biopak	Chardonnay	Kosovo	2010	187	N.D.
K19	Rahoveci	Merlot	Kosovo	2006	187	N.D.
K20	Shulina	Cabernet Sauvingnon	Kosovo	2010	187	N.D.
K21	Stone Castle	Red Wine	Kosovo	2011	750	N.D.
K22	Theranda	Barrique	Kosovo	2009	750	N.D.
K23	Erenik-Pavaresia	Merlot	Kosovo	1989-2001	750	N.D.
K24	Theranda	Pinot Blanc	Kosovo	2009	750	N.D.
K25	Suhareka	Gamay Noir	Kosovo	2010	750	N.D.
K26	Theranda	Gamay Noir	Kosovo	2009	750	N.D.
K27	Theranda	Italian Rhiesling	Kosovo	2009	750	N.D.
K28	Murati	Rose	Kosovo	2011	750	N.D.
K29	Shulina	Red Wine	Kosovo	2010	750	N.D.
K30	Bodrumi i Vjeter	Vranç	Kosovo	2012	750	N.D.
K31	Suhareka	Franconia	Kosovo	2012	750	N.D.
K32	Theranda	Rhine Rhiesling	Kosovo	2009	750	N.D.
K33	Theranda	Pinot Noir	Kosovo	2009	750	N.D.
K34	EKO	Red Wine	Kosovo	2012	750	N.D.
K35	Bodrumi i Vjeter	Cabernet Sauvignon	Kosovo	2011	750	N.D.
K36	Suhareka	Red Wine	Kosovo	2010	750	N.D.

Note. LOD = limit of detection, LOQ = limit of quantification, N.D. = not detected, HPLC-FD = High Performance Liquid Chromatography with Fluorescence Detection, OTA = Ochratoxin A

Table 2. OTA distribution in 18 analyzed bottled wine samples by HPLC-FD, imported in Kosovo.

Sample code	The name of the company	Variety	Country	Year of production	Volume of bottle/ ml	Content of OTA, ng/ml
A1	Luani	Merlot	Albania	2003	187.5	1.204
A2	Luani	Cabernet Sauvignon	Albania	2011	750	0.634
A3	Luani	Riesling	Albania		750	N.D.
M1	T'GA ZA JUG	Vranec	Macedonia	2009	187	0.331
M2	T'GA ZA JUG	Red Wine	Macedonia	2012	187	0.229
M3	T'GA ZA JUG	Vranac	Macedonia	2012	187	0.203
I1	Cantine del colle	Rosso	Italy		750	0.18
I2	Cielalo	Cabernet Sauvignon	Italy	2012	750	0.17
I3	Celine Casa Bottega	Chardonnay	Italy	2009	750	N.D.
F1	Cuvee Louis XII	Chardonnay	France		750	0.09
S1	Quercus	Merlot	Slovenia	2008	200	< LOD
E1	Ash Tree Estate-Freixfenet	Shiraz-Monastrell	Spain	2011	750	< LOD
AS1	Monty's Hill	Shiraz & Cabernet Sauvignon	Australia	2012	750	N.D.
B1	Mezzek	Merlot	Bulgaria		750	< LOD
B2	Yamantiev's	Cabernet Sauvignon	Bulgaria	2012	750	< LOD
B3	Saint Ilia-Tracia	Red Wine	Bulgaria	2009	750	N.D.
MN1	Plantaze	Cabernet Sauvignon	Monte Negro	2010	750	0.06
MN2	Plantaze	Vranac Pro Corde	Monte Negro	2010	750	0.05

Note. LOD = limit of detection, LOQ = limit of quantification, N.D. = not detected, HPLC-FD = High Performance Liquid Chromatography with Fluorescence Detection, OTA = Ochratoxin A

3.1. Discussions

It was determined that the amount of OTA in all analyzed 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 most of the samples analyzed, the amount of OTA is below the detection limit (LOD) or not detected (N.D.) at all. From the results obtained (tab. 1) we can see that in 36 wine samples produced and bottled in Kosovo, the amount of ochratoxin A is < LOD in 16 of them and in 20 of them the ochatoxin A is not detected at all. The results of the wine samples from other countries that import wine in Kosovo show that in some of the Albanian and Macedonian bottled wines that we have analyzed, OTA concentration is slightly higher compared to other countries of the Balkan, Europe and beyond (tab. 2). For example if we analyze the result of the sample encoded as A1 (tab.2) we can see that the amount of OTA in this wine produced in Albania, is quite high in the bottle although within the limits allowed by EU. We can see that from three bottled wines produced in Albania in two of them we have isolated OTA (tab.2). Also from the results we can see that the bottled wines produced in Macedonia, from a total of three analyzed bottles, in three of them is isolated OTA (tab.2). Regarding the analyzed bottled wines produced in Italy, in two of them is isolated OTA, although within the limits allowed by EU. The analyzed bottled wines from other countries shown to have a minimal or no presence of ochratoxin A (tab.2.). From the results obtained we can see that OTA is isolated in bottled wines produced in different years, confirming that each year in viticulture and winery has its own specifics, which besides the technological and sanitary conditions largely influenced by climatic factors of the respective year. Also from the results we can see that the concentration of OTA above the limit of detection is shown only to samples of red wine which verifies that the risk of higher concentra-

tion of OTA is expected to be in red wines than white wines (tab.1& tab.2) Research more or less similar are also made by our colleagues who determined that in the red wines OTA amount expected to be higher compared with white grape varieties. For example a study conducted in Slovakia (Belajova et al, 2007) where the bottled wines were also involved in research, shows the greater presence of OTA in red wines than white wines. Technological progress in the making of wine, which implies first the advanced hygienic sanitary conditions as well as the standard process during all other technological stages are seen to be a key factor in the minimum concentration of OTA in bottled wine (Durguti et al, 2014). Taken into consideration the fact that many of the consumers consume wine before bottling, we recommend the Enologists who deal with scientific work to analyze the amount of OTA since the initial stages of grape processing in order to verify whether the raw wine exceed or not the limits allowed by EU for ochratoxin A. We also recommend the wine technologists (enologist), especially those from the countries where prevailing temperatures slightly higher, to increase attention to cleanliness and all other technological aspects, conditions that favor the growth of the fungi responsible for the production of OTA. 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.

Conclusions

The importance of the obtained results lies in the fact that this type of research is performed for the first time in Kosovo (bottled wine) and as such provides harmless consumption of the wines produced and imported in Kosovo.

References

1. Arbizu, T., Amezqueta, S., Penas, E., de Cerian, A. (2010): Occurrence of Ochratoxin A in Southern Spanish Generous Wines under the Denomination of Origin "Jerez-Xérès-Sherry and 'Manzanilla' Sanlúcar de Barrameda" – Toxins, 2(5): 1054–1064.
2. Belajova, E.and Rauhova, D. (2007): Determination of ochratoxin Aand its occurrence in wines of Slovakian retail, Journal of Food and Nutrition Research Vol. 46, 2007, No. 2, pp. 68-74
3. Cabañes, F., Bragulat, M., and Castella, G., (2010) Ochratoxin A Producing Species in the Genus Penicillium, Toxins (Basel). 1111–1120.
4. Centre for Food Safety, Food and Environmental Hygiene Department,The Government of the Hong Kong Special Administrative Regiondried vine fruits, (2006): Ochratoxin A in Food, Report nr.23, http://www.cfs.gov.hk/.
5. Durguti, V., Georgieva, A., Angelov, A., and Bajrami, Z. (2014): Quantitative determination of ochratoxin A in wine after the clarification and filtration, Croatian Journal for Food Science and Technology, Vol.6 No.2, 79-83.
6. El Khoury, A., and Atoui, A. (2010): Ochratoxin A: General Overview and Actual Molecular Status, Toxins 2(4): 461–493.
7. Flajs, D., Domijan,A-M., Ivic, D., Cvjetkovic, B., Peraica, M. (2009): ELISA and HPLC analysis of ochratoxin A in red wines of Croatia, Elsevier, *Food Control* 20, 590-592.
8. Marquardt R.R., Frohlich A.A. (1992): A review of recent advances in understanding ochratoxicosis, PubMed J Anim Sci.70 (12), 3968-88.
9. Olivia, C., and Catara, V., (2011): Mycoflora and Biodiversity of Black Aspergilli in Vineyard Eco-Systems, The Dynamical Processes of Biodiversity - Case Studies of Evolution and Spatial Distribution, PhD Oscar Grillo (Ed.), ISBN: 978-953-307-772-7, InTech
10. Peraica, M., Flajs, D., Domijan,A.,Ivić, D., Cvjetković, B. (2010): Ochratoxin A Contamination of Food from Croatia, Toxins 2 (8), 2098-2105.
11. Visconti, A., Pascale, M., Centonze, G. (1999): Determination of ochratoxin A in wine by means of immunoaffinity column clean-up and high-performance liquid chromatography, Journal of Chromatography, 89-101.
12. Vukovic, G., Pavlovic, S., and Ristic, M.S. (2009): Comparasion of two sample preparation procedures for HPLC determination of ochratoxin A, Arch. Biol. Sci., Belgrade, 61 (4), 639-644.
13. Yordanova, P., Wilfried, K., Dimitrov, P. (2010), Ochratoxin A and β2 Microglobulin in BEN Patients and Controls, Journal List Toxins (Basel), V2 (4).
14. Zimmerli, B., Dick, R. (1996): Ochratoxin A in table wine and grape-juice: Occurrence and risk assessment. Food Additives and Contaminants 13, 655-668.