QUANTITATIVE DETERMINATION OF OCHRATOXIN A IN MUST

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Abstract

For quantitative analysis of ochratoxin A in must we have used the analytical method known as HPLC-FD and previously used the immunoaffinity clean-up procedure for extraction of ochratoxin A by high immunoaffinity columns. We have determined the quantity of OTA in 30 must samples. Samples that are analyzed have been taken in the vineyards of Kosovo, concretely in southern part of Kosovo, in Rahoveci and Suhareka region. The results of all analyzed samples have been below the limit allowed by 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: *must, ochratoxin A, HPLC-FD, immunoaffinity column, mycotoxin*

1. INTRODUCTION

The must grape represents the grape juice from which by means of certain technological processes, in particular alcoholic fermentation process created wine booze. A quantitative determination of ochratoxin A in must is very important to have in early stage of the technological process of winemaking an overview regarding the presence or not of substances dangerous to human health as is in this case the mycotoxin known as ochratoxin A.

Ochratoxin A, N-[(3R)-(5-chloro-8-hydroxy-3-methyl-1-oxo-7-isochromanyl) carbonyl]-L-phenylalanine, is a mycotoxin produced by certain species of Aspergillus and Penicillium filamentous fungi. The OTA levels in must and wine depend on different factors such as the climate, the date of harvesting and different wine-making procedures. 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 et al, 1992; Steyn et al, 1999).

Therefore, using musts with low OTA levels will be possible to produce wines with toxin levels below the limits set by the European Commission (EC) 2 μg/kg (Ponsone et al.2010).

This mycotoxin was detected particularly in red and dessert wines, in grape juice and musts as well (Belajova et al. 2007)

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 (Khoury, A., & Atoui., A, 2010). 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).

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 0.1 to 5.0 ng OTA/ml. The working standards were freshly prepared every day.

2. 2. *Sampling*

Must samples of 2014 were taken from two regions in Southern Kosovo, the only regions which different varieties of table and industrial grapes are cultivated in, and from different grape varieties different types of wine are produced. The must taken as a sample is held until the analysis in very cold temperature in order precluding the development of the yeasts and startup of the alcoholic fermentation process. Analyzing must has not been treated with any of the clarification substances. The most of the analyzed samples are industrial grape varieties and a part of the must samples are varieties of table grape. It's worth being mentioned the fact that from the two sampling regions (Rahoveci and Suhareka) in some cases for the comparative purposes have been taken for analysis the same grape varieties but from the various parcels of vineyards, for example sample nr. 8 and 12 (table 1). A total of 30 must samples were analyzed.The purpose of this research has been the quantitative determination of ochratoxin A in must, comparing the amount of ochratoxin A in must between two regions of Southern Kosovo, and all of this in very adverse climate conditions, which are characterized by frequent rainfall and and the absence of direct sunlight, necessary for the normal development of the grape-vine. Besides this, this research aimed to verify the possible risk or not from the consumption of grape and wine produced from these grape varieties by consumers.

2.3. *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 by means of immunoaffinity column clean-up and high-performance liquid chromatography. The must was first diluted with so-called extraction solution containing 1% polyethylene glycol (PEG 8000) and 5% sodium hydrogencarbonate, filtered to remove particulate matter 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.

2.4. *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.

3. RESULTS AND DISCUSSION

Nr. of sample	Region	Variety	Country	Year	Content of OTA ng / ml
1	Suhareka	Rhine Riesling	Kosovo	2014	N.D.
2	Rahovec	Vranac	Kosovo	2014	N.D.
3	Suhareka	Gamay	Kosovo	2014	N.D.
4	Suhareka	Italian Riesling	Kosovo	2014	< LOD
5	Rahovec	Cabernet Sauvignon	Kosovo	2014	N.D.
6	Suhareka	Muscat Hamburg	Kosovo	2014	< LOD
7	Suhareka	Franconia	Kosovo	2014	N.D.
8	Suhareka	Gamay	Kosovo	2014	N.D.
9	Suhareka	Pinot Nero	Kosovo	2014	< LOD
10	Rahovec	Gamay	Kosovo	2014	N.D.
11	Rahovec	Muscat Hamburg	Kosovo	2014	N.D.
12	Suhareka	Gamay	Kosovo	2014	N.D.
13	Suhareka	Pinot Blanc	Kosovo	2014	N.D.
14	Rahovec	Italian Riesling	Kosovo	2014	N.D.
15	Suhareka	Pinot Blanc	Kosovo	2014	N.D.
16	Rahovec	Žametovka	Kosovo	2014	N.D.
17	Rahovec	Žametkova	Kosovo	2014	< LOQ
18	Rahovec	Pinot Nero	Kosovo	2014	N.D.
19	Suhareka	Pinot Blanc	Kosovo	2014	N.D.
20	Suhareka	Rhine Riesling	Kosovo	2014	N.D.
21	Suhareka	Italian Riesling	Kosovo	2014	N.D.
22	Rahovec	Chardonnay	Kosovo	2014	N.D.
23	Rahovec	Muscat Italia	Kosovo	2014	N.D.
24	Suhareka	Sauvignon Blanc	Kosovo	2014	N.D.
25	Suhareka	Gamay	Kosovo	2014	N.D.
26	Suhareka	Pinot Nero	Kosovo	2014	N.D.
27	Rahovec	Žametkova	Kosovo	2014	N.D.
28	Rahovec	Vranac	Kosovo	2014	N.D.
29	Rahovec	Smederevka	Kosovo	2014	N.D.
30	Suhareka	Rhine Riesling	Kosovo	2014	N.D.

Table 1. *OTA concentration in 30 analyzed must samples by HPLC-FD*

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 2 ng/ml, concretely in all of the samples analyzed, the amount of OTA is below the detection limit (LOD) or not detected (N.D.) at all. The purpose of the research was the analysis of ochratoxin A in must, and in the case of detection of ochratoxin A, monitoring of its movement during subsequent alcoholic fermentation process.

As stated above, the vine cultivation weather conditions in these two regions have been extremely unstable, which are mainly characterized by frequent rains and lack of sunlight during the growth and maturation phase of different analyzed varieties of vine grapes.

The lack of ochratoxin A isolation in almost all samples shows for adverse climatic conditions, especially the lack of direct sunlight, one of the main necessary conditions for the growth of fungi responsible for the synthesis of the ochratoxin A, first in the grape berries and from there then in must and wine.

Besides the difficulties that arise in the winemaking process as a result of these climatic conditions, one thing is certainly positive that these analyzed must samples will produce the pure wine in the sense of the presence of ochratoxin A, as extremely hazardous substance for human health. This research do not shows the risk of drinking the wine produced from the must that we have analyzed in the future by consumers and as such do not represent a risk for human health.

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