QUANTITATIVE DETERMINATION OF OCHRATOXIN A DURING ALCOHOLIC FERMENTATION

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Abstract

Through the use of the immunoaffinity clean-up procedure for extraction of ochratoxin A by high immunoaffinity columns by means of the analytical methodology known as HPLC-FD we have analyzed the quantity of ochratoxin A in 22 must and wine samples during alcoholic fermentation process. Analysis of the samples was done in the University of Food Technologies, Plovdiv – Bulgaria.OTA quantities in all analyzed samples have been under the level allowed by EU for ochratoxin A i.e. 2 ng/ml and as such these wines after consuming do not pose a risk in the future to human health.

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

1. INTRODUCTION

The alcoholic fermentation process is the anaerobic process where essential role play the Sacharomyces yeasts, which appear to metabolise the sugar present in grape into ethyl alcohol (ethanol) and which process depends on a number of factors.

The rate of ethanol production from Sacharomyces varies by different factors, but can be $8x10^7$ -$9x10^7$ ethanol molecules per yeast cell per second (Fugelsang & Edwards, 2007).

During winemaking process there are various technological stages however undoubtedly the most important stage is the process of alcoholic fermentation.

Today for carrying out the alcoholic fermentation process in the best way, enologists usually prefer the use of commercial yeasts which allow a more efficient alcoholic fermentation process compared with spontaneous fermentation which occurs without the presence of commercial yeasts.

Dried commercial yeasts usually contain $1.1x10^{10}$-$3.9x10^{10}$ CFU / g (Fugelsang & Edwards, 2007).

Climatic conditions (high humidity and temperature) and geographical location are important factors favouring OTA accumulation in grape berries (Visconti et al, 2008).

It is known that OTA removal in white and red musts during AF occurs by two different phenomena: biodetoxification (microbial enzymatic hydrolysis of toxin molecule) and adsorption (Marco et al, 2012)

The latter is attributable to binding interactions with suspended solids, such as whole or fragmented grape skins, and/or yeast cell walls, which act like a sponge coated by negative charges that interact with toxin acidic feature (Marco et al, 2012).

Numerous scientific research points to an OTA steep reduction during alcoholic fermentation process (Visconti et al, 2008, Marco et al, 2012, Anabela et al, 2007 etc)

Warm weather conditions and rainfall favour the incidence of OTA production (Soma et al. 2012)

Temperatures of 30–35°C are optimal for A. carbonarius and A. niger growth, respectively, while OTA production is favoured by 20–25°C (Soma et al. 2012)

Regarding wine-making, preventive actions are to harvest early in high OTA risk areas when favorable conditions occur, segregate rotted bunches at harvest and minimize/avoid storage time before processing the grapes for wine making (Soma et al,2012).

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. 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 (Speijers et al, 1993).

The OTA levels in 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).

The uniqueness of OTA is its high stability. 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 (Muller, 1983).OTA is arguably a risk factor for Balkan endemic nephropathy (BEN).

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*

All must and wine samples were obtained from grape varieties of the vineyards of Agrokosova Holding Company, Suhareka-Kosovo, as well as monitoring of the fermentation process and sampling during fermentation are made in the same company. The alcoholic fermentation process is conducted in three wine tanks with three different varieties. Three types of analyzed must and wines were: Gamay Noir, Pinot Noir and White Wine (Italian Riesling, Rhine Riesling, Pinot Blanc). Regarding the white must the fermentation it is conducted at temperatures of about 12-18 C and is induced by special yeasts for white wine fermentation called SIHA White Arome (Sacharomyces Cerevisiae) produced by Begerow®Product Line. White grape must is treated with clarification enzyme (2 gr / Hl) prior to alcoholic fermentation process called SIHAZYM TM Claro produced by Begerow®Product Line. Regarding the red grape varieties the fermentation process is conducted at temperatures of about 23-25 C and are used the yeasts (Sacharomyces Cerevisiae) known as SIHA® Active Yeast 10 (Red Roman) produced by Begerow®Product Line and as such specific for red must fermentation. The quantity of yeast that we have used has been 20 g / Hl and during the alcoholic fermentation process we have used the food for yeast (30 gr/Hl) necessary for the normal development of the fermentation process, food that called SIHA PROFERM Plus (DAHP, ammonium sulfate and yeast cell wall preparation) produced by Begerow®Product Line. The samples taken during alcoholic fermentation process are stored in low temp until analysis. A total of 22 samples were analyzed and the aim of this research has been monitoring the movement of ochratoxin A if it already exists in must during alcoholic fermentation process and all of this in very difficult winemaking conditions as a result of adverse weather conditions.

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 and wine 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

The name of the grape variety	The day of fermentation	Country	Year	Content of OTA, ng/ml
Gamay Noir	1	Kosovo	2014	< LOQ
	2			< LOQ
	3			0.017
	4			< LOQ
	5			0.016
	6			0.021
	7			<LOD
	8			<LOD

Table 1. *OTA concentration in the grape variety Gamay Noir*

The name of the wine	The day of fermentation	Country	Year	Content of OTA, ng/ml
White Wine	1	Kosovo	2014	< LOQ
	2			< LOD
	3			< LOD
	4			N.D.
	5			N.D.
	6			N.D.
	7			N.D.
	8			N.D.

Table 2. *OTA concentration in the white must and wine (Italian Riesling, Rhine Riesling, Pinot Blanc)*

The name of the grape variety	The day of fermentation	Country	Year	Content of OTA, ng/ml
Pinot Noir	1	Kosovo	2014	< LOD
	2			< LOD
	3			< LOD
	4			< LOD
	5			N.D.
	6			N.D.
	7			N.D.

Table 3. *OTA concentration in the grape variety Pinot Noir*

Initially worth mentioning that the climatic conditions for normal growth and ripening of grapes have been extremely unfavorable, the conditions which are characterized by frequent rainfall and absence of direct sunlight, which have caused serious problems during winemaking process. The main problem was the lack of optimal quantity of sugar, an amount necessary for the normal development of the alcoholic fermentation process. The purpose of this research as we pointed out above has been

quantitative analysis of ochratoxin A in some of the grape varieties without knowing in advance the amount of OTA in the analyzed must and thus to be seen indirectly how these conditions have affected the growth and development of fungi responsible for producing of this kind of micotoxin. If we analyze the results of the variety Gamay Noir (table 1) we can see that since the beginning of the alcoholic fermentation process the amount of OTA has been under the limit of quantification (< LOQ) and in recent days it is reduced to under the limit of detection (< LOD). At variety Gamay Noir, the sample No. 6 (table 1) we can see a slight increase in the amount of OTA compared with the sample No. 5.This as a result of which only sample No. 6 is obtained from the sediment of the wine tank and not at the middle part of the wine tank as other samples. Although this difference is extremely small, shows a tendency to accumulation of OTA in the bottom of the wine tank and dumping out in the future through the racking process. Regarding the results of the analyzed white wine (table 2), we can see that at the beginning of alcoholic fermentation process the presence of OTA it was under the limit of quantification to be reduced under the limit of detection and finally in the end to be not detected at all. Results more or less similar are also obtained for the variety Pinot Noir (table 3). Even here at the beginning of the fermentation process the amount of OTA has been under the limit of detection to be not detected in the last days of the fermentation process. From this research we can see that despite the difficulties in the process of alcoholic fermentation as a result of adverse climatic conditions there is no risk in the future from the consumption of wine produced from these analyzed grape varieties. And in the end it seems that climatic conditions with more frequent rainfall and the absence of direct sunlight during development and ripening of the grape vine followed by frequent spraying with chemical preparations which contain fungicides does not favor the growth of fungi responsible for producing of ochratoxin A.

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