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ORIGINAL RESEARCH PAPER

THE PROBLEM OF OCHRATOXIN A IS REAL IN ARMENIA

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Abstract: Mycotoxins are especially dangerous biological contaminants of food. OTA (ochtatoxin A) is the main problem in countries with developed winemaking and viticulture.
The aim of our work was to identify contamination level of dried vine fruit by ochratoxigenic and aflatoxigenic filamentous fungi and their mycotoxins and to reveal dangers for consumer. Dried vine fruit sold in Armenia including local production and imported were analyzed. Quantities of OTA and AFB1 (aflatoxin B_1) in fungi extracts and in dried grape samples were determined by TLC (Thin-Layer Chromatography) method.
508 strains of potential producers of OTA and AFB1 from genus *Aspergillus* were isolated from dried grape and identified. 30 strains of *A. carbonarius*, 15 strains of *A. niger*, 3 strains of *A. ochraceus* and 12 strains of *A. flavus* were analyzed on mycotoxin content. OTA were revealed in 20 strains of *A. carbonarius* and 6 strains of *A. niger*.
In order to discover the sources of contamination of Armenian dried vine fruit by toxigenic species, mycological analysis of fresh grape berries was carried out. 218 potentially toxigenic strains were isolated and 43 strains (20 *A. carbonarius*, 16 *A. niger* and 7 *A. flavus*) were analyzed. None of them produced mycotoxins. OTA and AFB1 were revealed in dried grape samples of Armenian production.

Keywords: *aflatoxin B1, dried vine fruit, ochratoxin A*

INTRODUCTION

Ochratoxin A continues to attract global attention concerning the hazard and impact on both human and animals based on its toxicity and occurrence [1]. Aflatoxins, ochratoxins and patulin are among the greatest economic damage causing mycotoxins which are contaminating fruits and vegetables. Producers of these mycotoxins are filamentous fungi mainly from the genera *Aspergillus* and *Penicillium*. Contamination of fruit crops including grapes, by filamentous fungi occurs in different stages of their growth.

Grape is the subject of fungal contamination during cultivation, harvest, transportation and storage [2]. Species from *Aspergillus* section *Nigri,* considered as the main fungi responsible for the infection of grape varieties with ochratoxin A, can be isolated in the late stages of berry maturation in pre-harvest period [3]. In general, *A. carbonarius* is considered as the most important ochratoxigenic species in grapes due to its common occurrence and its high production ability (almost all of the strains are able to produce the toxin at different levels) [3].

Controlling the level of grape contamination by species *A. carbonarius* is particularly important in regions with prevailing wet climate conditions [4]. Studies have shown that temperature and humidity are the main environmental factors directly affecting the growth of fungi and the synthesis of mycotoxins before and after harvest [5]. In Armenia the presence of OTA in grapes can be explained by the high content of sugars, which contributes to the growth of ochratoxigenic species *A. carbonarius* and OTA synthesis [6].

The use of grapes that contains OTA for the preparation of juices and wines may be a concern. After grape, juices, wines and dried vine fruit, are the largest sources of exposure to OTA. Dried vine fruit (raisin, sultana and currant) is the second (after wine) most important product of viticulture [3]. Concerning this, the contamination of dried grape by ochratoxigenic fungi and ochratoxin A attracted much attention. Dried fruits, including dried vine fruits, are susceptible to contamination by fungi and mycotoxins, since they contain a sufficient amount of moisture and sugars [3].

OTA is a mycotoxin classified as a possible human carcinogen (group 2B) by the International Agency for Research on Cancer [7]. Because of its toxic properties (nephrotoxic, genotoxic, immunotoxic and teratogenic) and ability to accumulate in the kidneys the Commission Regulation EC has set limits for OTA in wine, grape juice, grape nectar, grape must and concentrated grape must at 2.0 $\mu g \cdot kg^{-1}$, and in dried vine fruits - at 10 $\mu g \cdot kg^{-1}$ [8].

Armenians were one of the ancient peoples of the world who had developed viticulture and produced wine and dried grapes. The Phoenicians and Armenians traded raisins with Greece and Rome in 1200 - 800 B.C. [9]. But nowadays Armenia is not mentioned as a country with developed viticulture and winemaking. One of the reasons is the problem of entering the international market. Small farms on the production of grapes, wine and other grape derived product, are not able to provide a stable supply of quality products to the European market. However, Armenia has a real potential for this.

In this regard, the development of a strategy to increase the production volumes of grapes and grape derived products and compliance their quality with international standards is one of the important economic tasks that can ensure the sustainable

development of this industry. To achieve this, the primary challenge is the need to create a scientifically validated database on biological safety of the products.
The aim of our work was to identify contamination level of dried vine fruit by ochratoxigenic and aflatoxigenic filamentous fungi and their mycotoxins (OTA, AFB1) to reveal dangers for consumer.

MATERIALS AND METHODS

Mycological analysis of dried vine fruit and grape berries

Sampling

Totally 167 samples of dried vine fruit and 81 samples of fresh grape were analyzed. Sampling was done in accordance with EC Regulation 401/2006 [10]. Grape samples were collected from vineyards in Ararat (20 samples), Armavir (23), Kotayk (18) and Vayotsdzor (20) regions during their technological ripeness and harvesting period. 167 Dried vine fruit samples were taken from different markets and supermarkets of Yerevan (147 samples) and some manufactures in Armavir region (20 samples).

Isolation of filamentous fungi from analyzing samples

Mycological analyses of samples were carried out with direct planting and serial dilution planting methods [11]. An average sample was prepared. Each sample was made up of around 3 kg of dried grape and fresh grape berries. 10 g of material was taken and homogenized. And subsequent dilutions were done. Dilution included the following steps: 1) shaking the product suspension for 15 minutes on a shaker (Stuart SSL2, UK); 2) infusion for 10 minutes; 3) preparation of serial dilutions 1:10 and 1: 100; 4) surface plating from dilutions on nutrient mediums CYA (Czapek-Yeast Agar medium, HiMedia Ltd.) and GYA (Glucose-Yeast Agar medium, HiMedia Ltd.). Plates were incubated at 25 ^{o}C for 7 days.

Identification of fungi species

Identification of isolated cultures of filamentous fungi was done based on macroscopic and microscopic characteristics by manuals cited in brackets [12]. For identification of species from section A*spergillus Nigri* cultures were grown on following nutrient mediums: CYA (Czapek-Yeast Agar medium, HiMedia Ltd.), GYA (Glucose-Yeast Agar medium, HiMedia Ltd.), MEA (Malt-Extract Agar medium, HiMedia Ltd.), SDA (Sabouraud Dextrose Agar, HiMedia Ltd.), at 25 ± 1 ^{o}C and 37 ± 1 ^{o}C.

Toxicological analyses of isolated fungi strains and dried grape samples

Ochratoxin A and aflatoxin B_1 extraction from fungi strains

Cultivation of the tested fungi: Isolates of fungal species were screened for toxicity and mycotoxin production. Cultivation of fungi was carried out by a surface method on Czapek-Dox liquid medium. Incubation of strains was carried out at a temperature of 28 - 30 °C, for 10 - 14 days [13]. After growth of fungi the contents of flask (culture liquid + mycelium) were homogenized in 50 mL chloroform and extracted.

Extraction procedures: The contents of each flask (mycelium + medium) were homogenized in 50 mL chloroform for 5 min in a high speed blender (Redmond RHB-2920-E) (16000 rpm).
Extraction was repeated three times. The combined chloroform extract was filtered over anhydrous sodium sulphate, (5g) evaporated to near dryness using a rotary evaporator (IKA RV 10, Germany), and the dry material was transferred to a dry vial with small amount of the solvents which was evaporated to near dryness under the air.

Extraction of dried grape samples

The selected samples were ground for 1 - 2 minutes in laboratory blender (Redmond RHB-2920-E, Redmond Industrial Group LLC, Chine). For determination of OTA 25 g of crushed product was placed in a conical flask in volume of 250 mL. 12.5 mL of 1 % solution of acetic acid in water and 125 mL of chloroform were added and shacked for 30 minutes. The mixture was filtered through a folded paper filter. 50 mL of the filtrate was taken [14].
For the determination of AFB1 25 g of crushed product was placed in a conical flask. 25 mL of 10 % solution of sodium chloride in water and 125 mL of acetone were added and shacked for 30 minutes [15].

Clean-up of extracts for TLC

Clean-up for OTA determination: Clean-up was done according to Galvis-Sanchez and Barros [16]. 50 mL of filtrated extract was shaken with 35 mL of 3 % sodium hydro carbonate and methanol-water mixture (in ratio of 1:4) in separatory funnel. After separation of layers the upper water layer was set apart and the lower chloroform was extracted (2 times) with 35 mL of 3 % sodium hydro carbonate. The combined water extracts were shaken with chloroform (2 times with 25 mL), each time after separation the lower chloroform layers were thrown away. The water extract was acidified with water solution of sulfuric acid with concentration 2 $mol \cdot L^{-1}$ till *p*H is 2 - 3. Water solution was extracted with chloroform (1 time with 50 mL and 2 times with 25 mL) in separatory funnel. The combined chloroform extracts were dried with anhydride sodium sulfate (10 - 12 g) in 30 min. The solution was filtered through cotton and evaporated on rotary evaporator at temperature of 40 - 45 ^{o}C. The residue was dissolved in mixture benzene - acetic acid (99:1) for TLC application.
Clean-up for AFB1 determination: 20 mL of 15 % lead acetate solution and 30 mL of distilled water were added to 50 mL of filtrate, stirred and left for 10 minutes in dark. The formed precipitate was filtered through a folded paper filter. 80 mL of filtrate was shaken in a separatory funnel with hexane (2×30 mL) each time discarding the upper hexane layer. The water-acetone layer was extracted with chloroform (1 time 40 mL, 2 times 30 mL with chloroform - acetone mixture (3:1)). The combined chloroform extracts were placed in a flask of 250 mL. In order to remove the water 5 - 7 g of anhydrous sodium sulfate was added, shaken and left for 30 min in dark. The dried solution was filtered through a chemical funnel with a cotton ball. Sodium sulfate was washed with 10 mL chloroform and wash added to the filtrate. The chloroform extract was evaporated to dryness on a rotary evaporator. The residue was dissolved in benzene - acetonitrile mixture (98 : 2) and analyzed by TLC [15, 16].

Determination of mycotoxins by Thin-Layer Chromatography method

For preliminary qualitative determination of OTA and AFB1 two-dimensional thin-layer chromatography was employed [17]. For carrying out of TLC the plates with aluminum foil backing (Silufol, 10 cm x 20 cm) were used on which silica gel matrix was applied. *Determination of ochratoxin A:* Following mobile phases were used: ether – hexane – chloroform – formic acid (30:30:30:1) for the first direction; and toluene – ethyl acetate – formic acid (55:35:10) for the second direction.

The Silufol plate was marked with thin pencil lines. 20 µL of sample extract was applied in the lower right corner at a distance of 1.5 cm from the edges. In the upper right corner 3 µL (15 ng) of working OTA solution was applied. 1 µL, 3 µL and 5 µL (5, 15 and 25 ng respectively) of the working solution of OTA were applied in the lower left corner at a distance of 1 cm, 2 cm and 3.0 cm from the left edge and 1.5 cm from the lower edge of the plate. After two-dimensional TLC the plate was removed from the chamber and dried. The plate was sprayed with saturated sodium bicarbonate solution. In the presence of OTA the color of the spots changes from blue-green to blue. The quantitative determination of OTA was carried out in accordance with GOST R 52828-2007 [14] using a Sorbfil densitometer (UV 254; UV 366).

Determination of aflatoxin B1: AFB1 determination in *A. flavus* strains was also carried out by TLC analysis. The method differs only in used reagents. Following mobile phases were used: a mixture of ether – methanol – water (94:4.5:1.5); and chloroform – acetone - water (90:10:1) for the second direction [17].

The plates were sprayed with HNO_3:H_2O solution (1:3). The quantitative determination of OTA was carried out in accordance with GOST R 30711-2001 [15].

RESULTS AND DISCUSSION

Mycotoxins in extracts of fungi strains isolated from dried and fresh grape

Dried vine fruit sold in Armenia including local production and imported were analyzed. The results have shown high contaminant level of dried grape by toxigenic fungi from *Aspergillus* genera. 508 strains - potential producers of OTA and AFB1 belonging to genus *Aspergillus: A. carbonarius, A. niger, A. sclerotioniger, A. ochraceus, A. lacticoffeatus* and *A. flavus,* were isolated from 167 dried grape samples (white and black varieties) (Table 1).

Table 1. *The quantity of toxigenic strains, isolated from grape and dried vine fruit*

Substrate		***A. carbonarius***	***A. niger***	***A. sclerotioniger***	***A. lacticoffeatus***	***A. ochraceus***	***A. flavus***
Armenian raisin	White	20	20	17	-	-	8
	Black	47	32	44	28	6	20
Armenian sultana	White	21	9	-	-	-	5
	Black	19	18	-	-	4	12
Imported raisin	White	10	18	-	-	-	5
	Black	23	37	-	-	-	10
Imported sultana	White	12	13	-	-	-	2
	Black	21	22	-	-	-	5
Total		173	169	61	28	10	67
Grape berries		98	96	9	-	-	15

During analysis of 81 fresh grapes 218 potentially toxigenic strains from genus *Aspergillus* were isolated and identified (Table 1).

Contamination of dried grapes by *A. carbonarius* is the major problem in connection with biosynthesis of ochratoxin A at different stages of dried grape production. The results of scientific studies conducted in several countries have shown that the detection of ochratoxin A in viticulture products, including dried grapes, is primarily associated with the presence of *A. carbonarius* species [18, 19].

The mycotoxin production of 103 strains isolated from both dried and fresh grape samples belonging to genus *Aspergillus* was investigated. 30 strains of *A. carbonarius*, 15 strains of *A. niger*, 3 strains of *A. ochraceus* and 12 strains of *A. flavus* from all analyzed were contaminants of dried grape. 20 strains of *A. carbonarius* were isolated from Armenian samples and 10 from imported. As shown in Figure 1, OTA was revealed in 20 strain extracts of *A. carbonarius* from 30 analyzed, in amount of 25.5 - 200 µg/100 mL.

The level of OTA production by strains contaminating Armenian dried grape samples was higher than in strains isolated from imported samples: 25.5-200 µg/100 mL and 45-55 µg/100 mL, respectively. This can be explained by high content of sulfur dioxide in imported samples which inhibit not only the growth of filamentous fungi but also mycotoxin production by them [20].

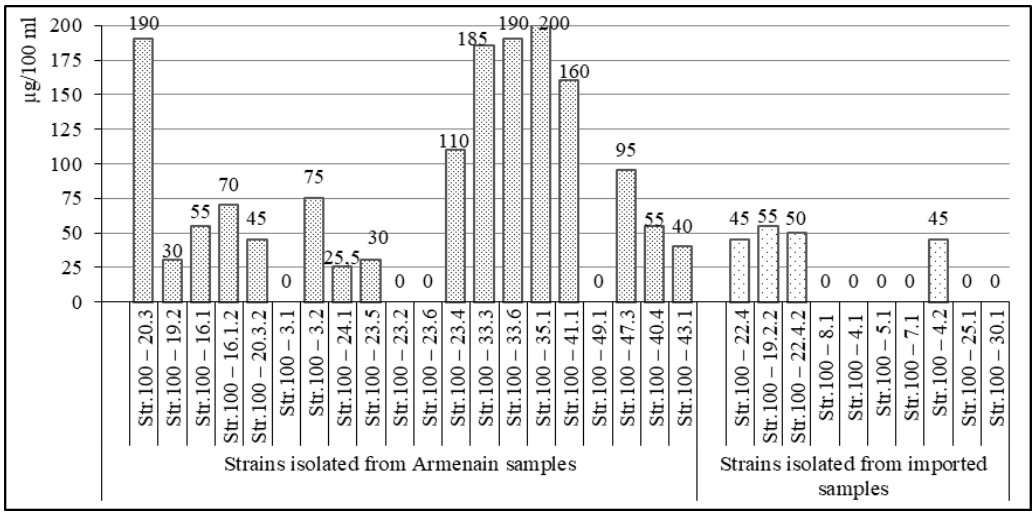

Figure 1. *OTA content in extracts of A. carbonarius strains isolated from different varieties of dried vine fruit*

A TLC analysis of 15 *A. niger* and 3 - *A. ochraceus* strains isolated from black varieties of dried vine fruit was carried out. OTA was detected in 6 *A. niger* strains extracts (3 of which from Armenian samples and 3-from Iranian). The concentration of OTA was 10-25 µg/100 mL in strain extracts from Armenian samples and 5 - 8 µg/100 mL from Iranian samples (Figure 2).

Despite the fact that *A. ochraceus* species is known as an active producer of OTA in food, this toxin was not revealed in any of the analyzed extracts of *A. ochraceus*.

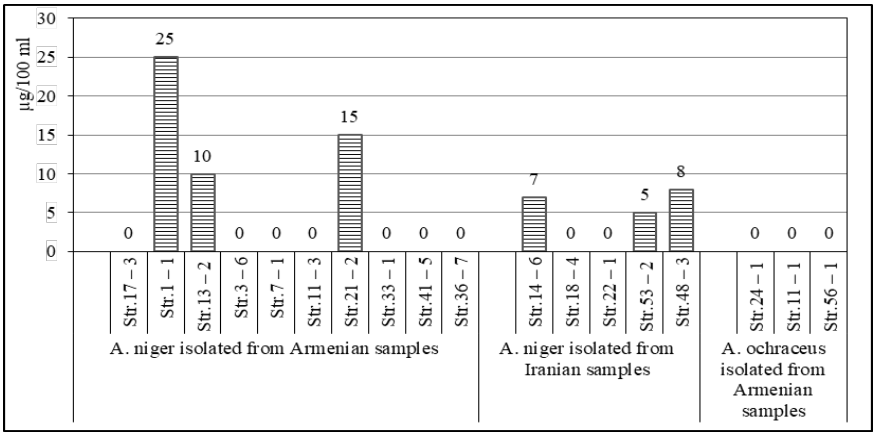

Figure 2. *The concentration of OTA in extracts of A. niger and A. ochraceus strains isolated from Armenian and Iranian samples*

During our analyses we have noticed a significant problem of dried vine fruit contamination by aflatoxigenic fungi and aflatoxin B_1 in Armenia. Recently, contamination of dried grape with the aflatoxigenic species *A. flavus* has been repeatedly reported [21, 22]. In most cases *A. flavus* is considered one of the most commonly found species polluting dried grape after *A. niger* and *A. fumigatus* species.
As a result of analysis 67 strains of *A. flavus* were isolated from dried grape. In 12 strains aflatoxin B_1 content was detected. Eight strains produced aflatoxin B_1 in quantities of 30 - 300 µg/100 mL (Figure 3).

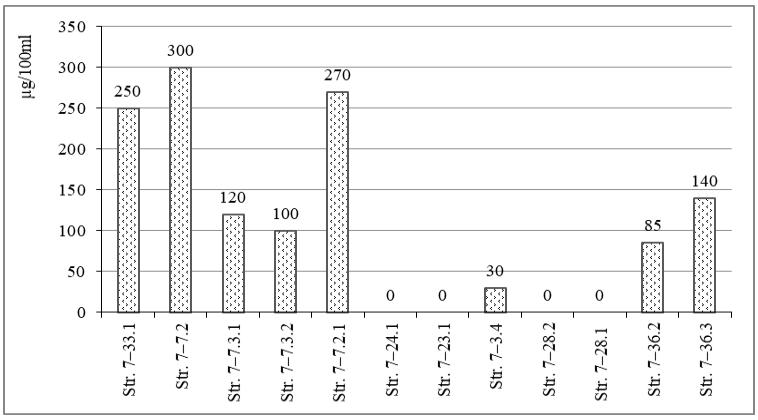

Figure 3. *AFB1 level in extracts of A. flavus strains isolated from Armenian black raisin*

In order to identify the sources of contamination of Armenian dried vine fruit by toxigenic species, mycological analysis of fresh grape berries from vineyards of the following regions in Armenia: Ararat, Armavir, Kotayk and Vayotsdzor, were carried out. The investigation of 81 samples of white and black varieties of fresh grape berries cultivated during the harvest season have shown high frequencies of occurrence of ochratoxigenic species. *A. carbonarius* and *A. niger,* dominated in all studied regions. Nine species belonging to *Aspergillus Nigri* section: *A. niger, A. carbonarius, A.*

sclerotioniger, *A. acoulaetus, A. foetidus, A japonicas, A. uwarum, A. pulverulentum* and *A. tubingensis* were identified (Figure 4). The results have shown that the main sources of contamination of dried vine fruit by *A. carbonarius* and *A. niger* ochratoxigenic species are grape berries. *A. flavus* species had an average frequency of occurrence.

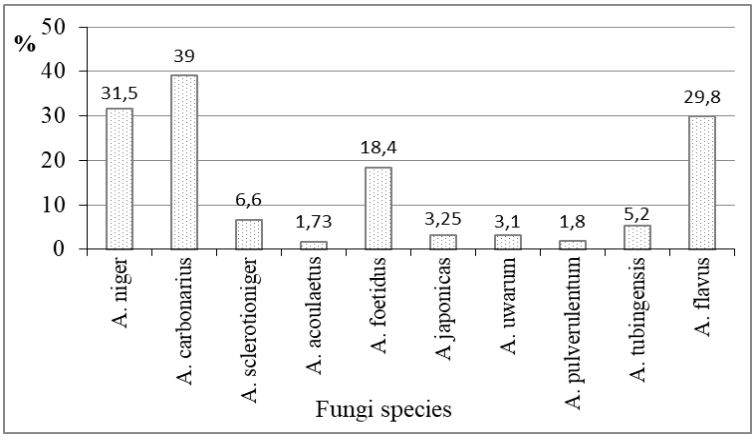

Figure 4. *Isolation frequency of species from Aspergillus section Nigri contaminating Armenian fresh grape berries*

According to Hocking et al. [23] the main sources of contamination of dried grapes, wine and other grape products by *A. carbonarius* and *A. niger* species are the soil and its remains on the grape berries. Contamination of grape berries mainly occurs due to the presence of mechanical damages on the skin of berries through which filamentous fungi penetrate inside. In ripe berries, with a low *p*H and high sugar content, more favorable conditions for the growth of fungi are created [3].
Despite the average frequencies of occurrence of toxigenic species, toxicological analyzes of 43 strains (20 *A. carbonarius*, 16 *A. niger* and 7 *A. flavus*) isolated from fresh grape samples have shown negative results. None of them produced OTA and AFB1.

Mycotoxins in dried vine fruit samples

Dried grapes pose a greater risk from the point of view of contamination with OTA, since during drying of grapes the relative proportion of *A. carbonarius* increases in the black aspergillus population due to decreasing of humidity [24]. There are numerous studies in which it is noted that the presence of ochratoxigenic fungi in dried grapes was accompanied by the presence of OTA in samples [18, 19]. Sometimes not only OTA but also AFB1 were found in raisin samples [25 – 27].
30 black dried grape samples (raisin and sultana) of Armenian production were investigated for the presence of OTA and 20 samples for AFB1. OTA was determined in 21 samples in amount of 10 - 45 $\mu g \cdot kg^{-1}$ (Table 2).
The presence of ochratoxin A in the studied local samples of black varieties is explained by their high contamination degree with the ochratoxigenic species *A. carbonarius* and *A. niger* [28].

Table 2. *The content of OTA and AFB1 in samples of black varieties of Armenian dried vine fruit*

Amount of analyzed samples	OTA not detected in	Quantity of OTA [μg·kg^{-1}]	Amount of analyzed samples	AFB1not detected in	Quantity of AFB1 [μg·kg^{-1}]
12 sultanas*	3 samples	10 - 25	8 sultanas	4 samples	7 - 18
18 raisin**	6 samples	23 - 45	12 raisin	4 samples	16 - 28

* sultana is mad from seeded varieties of grape
** raisin is mad from seedless varieties of grape

AFB1 revealed in 12 samples of all 20 analyzed in amount of 7 - 28 μg·kg^{-1}.
These analyses have shown that in Armenia there is not only a risk of contamination of dried grapes with ochratoxigenic species but also with aflatoxigenic species *A. flavus* and its mycotoxins. Black varieties of dried grape are particularly susceptible to the contamination because of the technology which is used for their production [28].

CONCLUSION

The analyses revealed that the main contaminants of dried grape are species of filamentous fungi from section *Aspergillus Nigri.* At the same time, ochratoxigenic species *A. carbonarius* and *A. niger* are dominated both in local and imported samples, the frequencies of occurrences of which were 73 % and 59 % in Armenian, and 44 % and 67 % in imported samples respectively. Armenian dried grapes were more contaminated by *A. carbonarius* than imported samples. While imported samples are more susceptible to contamination by *A. niger* species. On the other hand, imported samples content high amount of sulphur dioxide which is additional risk for human health.
OTA exceeded the limit established by EU Commission (10 μg·kg^{-1}) [8] in 70 % of Armenian samples from 30 totally analyzed. And the amounts of aflatoxin B1 (7 - 28 μg·kg^{-1}) in the 60 % of studied dried grape samples of Armenian production exceed the maximum permissible levels (2 μg·kg^{-1}) [8]. The high content of these mycotoxins is a great risk to consumer health.
Contamination of dried vine fruit by species from *Aspergillus Niger* section mainly occurs during the cultivation of grape: at ripening and harvesting stages, when possibility of mechanical injuries on the skin of grape berries is higher. Reasons of these damages could be insects, fungal pathogens, excessive irrigation or rain.
The use of appropriate agrotechnical measures and fungicides in grapes cultivation can significantly prevent the growth of fungi and synthesis of OTA. It is important to determine the date of harvest taking into account the forecasted weather conditions and organize harvesting in the right way which excludes mechanical damage of grape berries.
Secondary contamination can occur during poor sorting and drying process, in case of violations of drying regimes and non-compliance with hygiene conditions in the production area. In case of open air–solar drying climatic conditions play an important role. In rainy weather the likelihood of secondary contamination of the intermediate product with toxigenic fungi increases.
It is also essential to establish the limits of ochratoxigenic species in grape and grape products in EU Commission Regulations and the documents of Codex Alimentarius.

The control of amount of toxigenic fungus will be the best way to avoid the presence of OTA in these products in future.
The results of our investigations can serve as a good base for legislative bodies on food safety in the Republic of Armenia to make appropriate corrections in Rules and Regulations in accordance with EC Directives.

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