Fungal contamination of dried vine fruits and ochratoxin a detection in grape juice from Duhok, Iraq

Contaminación fúngica de frutos secos de vid y detección de ocratoxina en jugo de uva en Duhok, Iraq

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SCIENTIFIC RESEARCH

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ABSTRACT

Introduction: Dried vine fruits (raisins) and their juice are widely consumed by human as a diet. Raisins have been shown highly contaminated with ochratoxin A (OTA) and OTA-producing fungi. Ochratoxin A is a potent nephrotoxic and carcinogen to human and animals. **Materials and Methods:** Dried vine fruit samples was obtained from local shops for fruit juice and soft drinks in Duhok province**.** Two different media, Dichloran R ose Ben gal Chloromphenicol Agar (DRBC) and Dichloran 18 % Glycerol Agar (DG-18) was used for the counting and isolation of fungi from dried vine fruits. Grape juice were prepared from dried vine fruit after blending with water in a commercial blender. Natural contamination with ochratoxin A was detected by LC-MS/MS technique. **Results and Discussion:** All samples confirmed to be contaminated with fungi with various degrees. A total of 19 filamentous genera of fungi as well to Yeasts and non-sporulation mycelium was detected. Predominant genera detected on both media were *Aspergillus* and *Penicillium*. Detected value of ochratoxn A in juices obtained from dried vine fruits was between 0.37 ng/ml to 1.85 ng/ml. Samples contaminated with ochratoxin A were associated with *Aspergillus carbonarius, A. niger* aggregate, *A. sclerotium, A. ochraceus,* and *Penicillum verrucosum*. **Conclusion:** Dried vines fruit were highly contaminated with a broad spectrum of filamentous fungi. Black aspergilli were the most detected species from samples naturally contaminated with ochratoxin A.

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INTRODUCCTION

Ochratoxin A (O T A) is a secondery toxic metabolite secreted by some species of *Aspergilleus* sections *Nigri*, *Circumdati* and *Flavi* [1, 2, 3] and *Penicillium* belonged to sections *Penicillium* and *Furcatum* [4]. Ochratoxin A is a vigorous nephrotoxic mycotoxins that has been linked to problems of kidney in both human populations and livestock [5]. Ochratoxin A has been classified as a possible humans carcinogens (group 2 B) by the Internationel Agencies of Researches on Cancer (IARC) [6]. Ochratoxin A has been detected from various plant products such as grapes [7, 8, 9, 10], dried vine [11, 12], grape juices, musts and wines [13, 14, 15] coffee [16] and figs [17].

According to studies by several authors, strains from *Aspergilleus carbonarius* isolated from grapes and grapevine products showed a high percentage (75 -100 %) for ochratoxin A production [12, 18, 19]. The aim of the present study was to survey the fungal contaminated dried vine fruits and to determine the level of ochratoxin A in grape juice.

MATERIALS AND METHODS

Source of samples

Twenty samples of dried Vines fruits (used for production of grape Juices) were purchased from local shop for soft drinks and fruit juice in Duhok province during August 2010. The minimal size of each samples was 500 grams. All samples were stored in sterilized paper bags and stored in refrigerator at 5 ºC. Sample were processed during one week after collecting.

Isolation of fungi from dried vine fruits samples

Two different media, Dichloran Rose Bengal Chloromphenicol Agar (DRBC) (Fluka, Germany) and Dichloran 1 8 % glycerol agar (DG18) [20], were used for the isolation of the fungi from dried vine fruits. Sixty dried vine fruits chosen randomly from each sample, 30 of them were treated with 2 % of Sodium hypochlorite solution for 2 minutes after that rinsed with sterilized water and 10 fruits were aseptically placed in Petri plates with DRBC medium. Thirty dried fruits (10 per plate) were put directly on sterilized filter papers in plates (non –disinfected). Same number of dried fruits (disinfected and non-disinfected) were plated on DG18 medium. All the plates were incubated for seven days at 25 ºC in darkness.

Identification of fungi

Samples from dried vine fruits were examined daily with the help of stereomicroscope for sporulating Fungi. Pure colonies were confirmed on suitable medium for identification. Majority of detected fungi were identified to generic level depending on cultural and morphological features according to relevant manuals [20, 21]. Isolates from the genus *Aspergillus* were identified to species level according to the descriptions and keys provided by [1, 22, 23, 24].

Grape juice preparation and Ochratoxin A extraction

The preparation of grape juice was performed according to MacDonald *et al.*, [11], all dried vine fruits (5 0 gram) with four parts sterilized distilled water (250 m l) were blended at high speed in a commercialy blender for ten minute , with one minute repose each three minute, for prevention heating of sample. Then 3 ml. of the Juice was taken after passing through Millipore filter (0. 22) µm (Millex GP Filter Unit Coringhwhelton Co. Ireland) then mixed with 7 ml. of methanol in sterilized Eeppindrofs vial and stored in refrigerators for ochratoxin A detections.

Determination of Ochratoxin A by High performance liquid chromatography (HPLC)/ Tandem mass spectrometry L C- M S/M S

This was done at Princess Haya Biotechnology center, University of Science and Technology, Jordan. Chromatographic separation and M S detection was performed by using an Agilent 1200 Rapid resolutions (LC) and a 6460 Triple Quadruples Mass spectrometers. Then the samples were injected directly and analyzed without any further samples preparations.

For the L C technique 5m vMoL ammonium Acetate (pH: 3. 2) and Methanol were used as a mobile phase in Gradient modes. The column (ACE 5 C (18) (100 * 2.1 mm) was kept at 50 °C with a flow/ rate of 0.4 ml / minute. The total analysis time was set on 25 minute. An ESI source with Agilent Jet streams technology were coupled to the Mass Spectrometer.

The determination of an Optimal MRM transitions of all analyses were carried out using flow injection analysis of standard at the concentration levels around 2 .0 part per billion using Mass optimizers, an automated M R M technique developments Software.

RESULTS AND DISCUSSION

The contamination percentage of the twenty dried vine fruits samples by fungi both before and after surface disinfection on DRBC and DG18 media is presented in Figs.1and 2. Percentage of dried fruits with fungal infection both before and after surface disinfection is ranging between 30-100% and 16.67-90% respectively on DRBC medium, whereas, percentage of fruits with fungal infections before and after surface disinfection is ranging between 32.33-86.67% and 16.67-73.33% respectively on DG18 medium. The results revealed that a highest levels of fungi contaminations was detected in most of samples. Although surface disinfection reduced generally the numbers of dried fruits with viable moulds, there was a significant large internal mould invasions.

In a study on contamination of dried Vine Fruits with fungi, the percentage of fungal infection before and after the surface disinfection was established between 0-100% and 1-100% respectively [19].

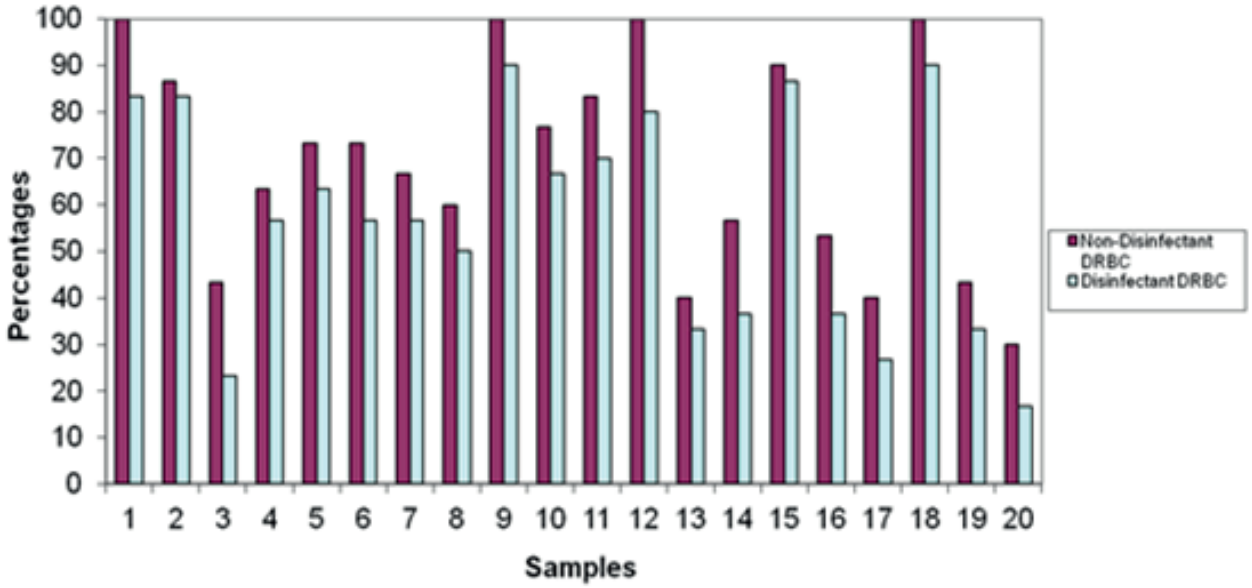


Figure 1. The percentage of dried Vine Fruits contamination with Viable moulds on DRBC medium

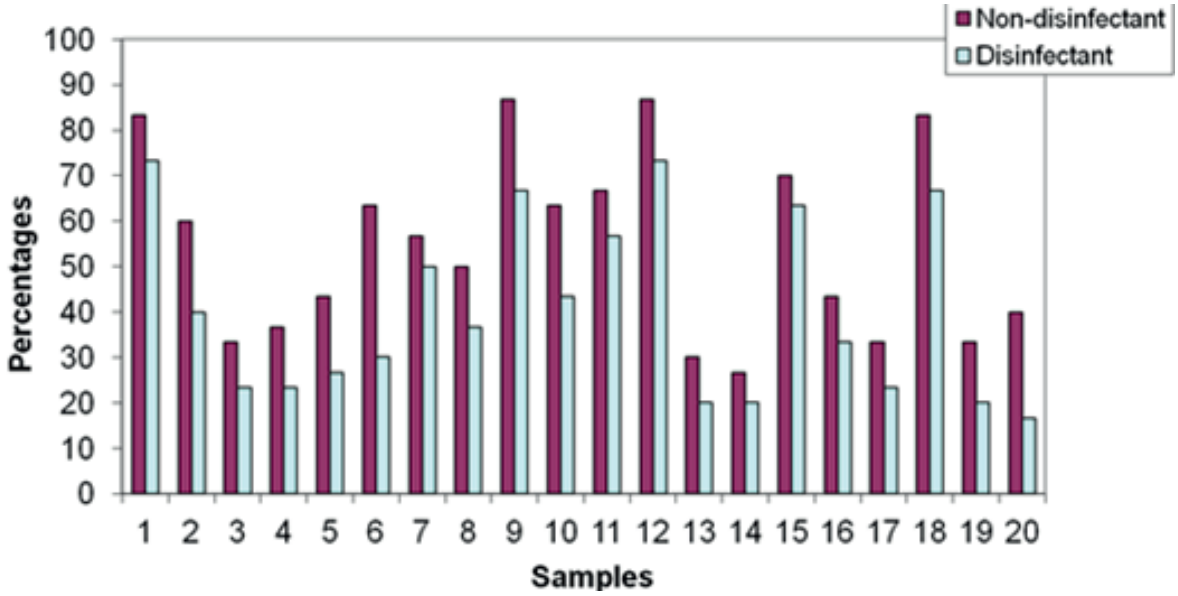


Figure 2. The percentage of died Vine Fruits contamination with Viable moulds on DG18 medium

The fungal genera isolated from samples after surface disinfection on DRBC and DG18 media are shown in Figs.3, 4. A total of 19 filamentous genera in addition to yeasts and non-sporulating mycelia were detected. Predominant on both media were *Aspergillus, Penicillium* and *Eurotium*. The predominance of *Aspergillus* in dried fruits was expected because members of this genus can survive drying process due to the relative resistant of their spores to sunlight and UV radiation, in addition to their ability of production of sclerotial resistant propagules [19, 22]. *Eurotium* species can grow exceptionally well at low water activity. They are therefore, common in foods with high concentrations of sugar [20]. Other relatively frequent contaminants were *Alternaria, Rhizopus, Trichoderma*, non-sporulating mycelia and yeasts. Similar result was found by [19].

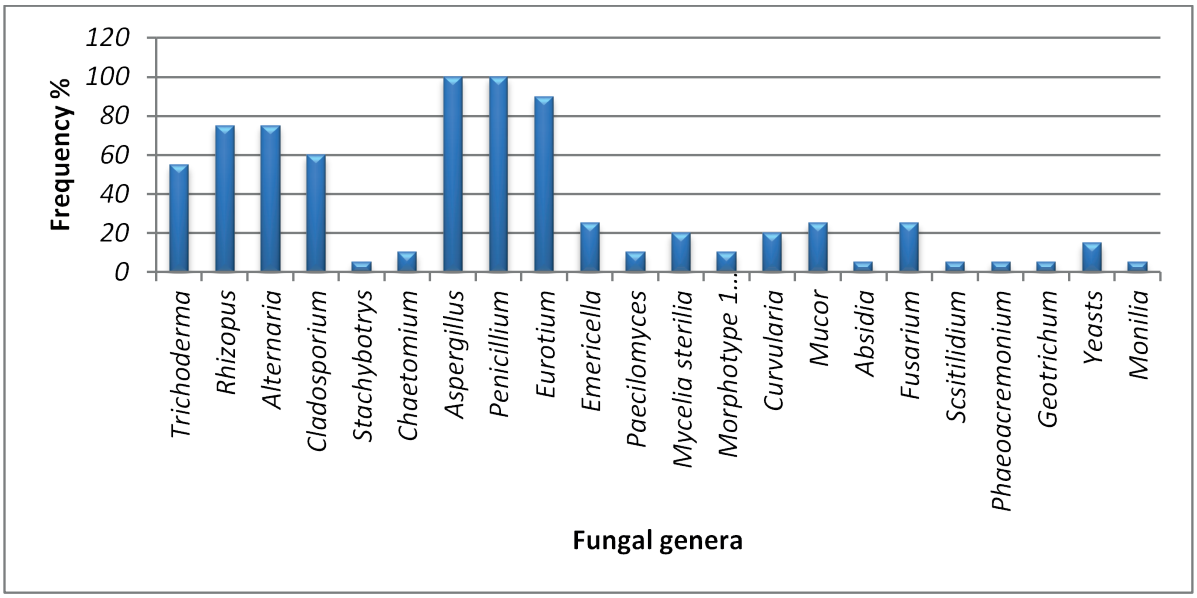


Figure 3. The frequency of fungal genera isolated from dried vine samples on DRBC

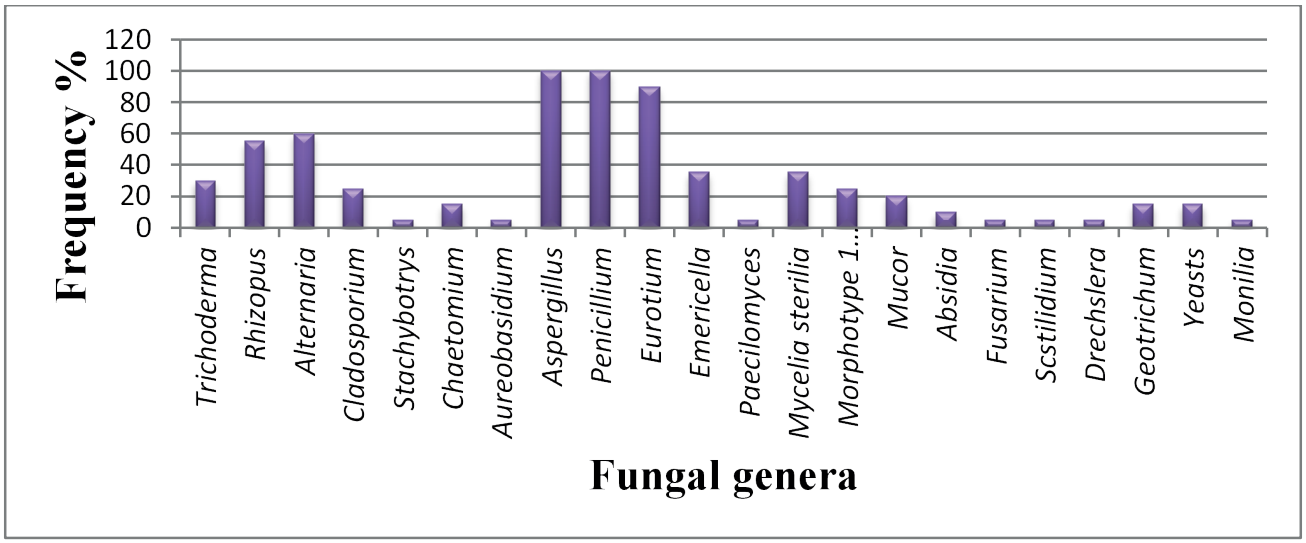
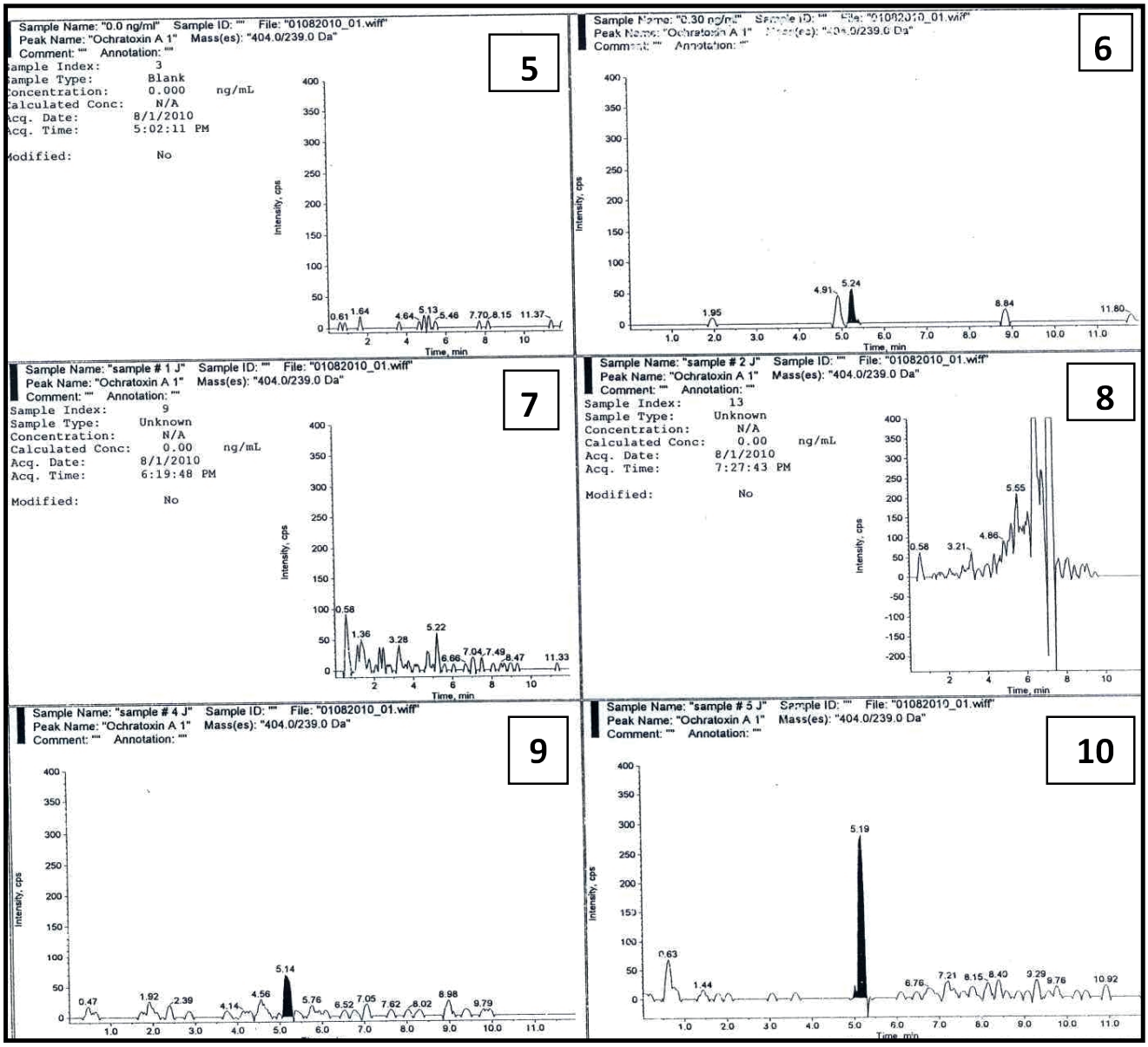


Figure 4. The frequency of fungal genera isolated from dried vine samples DG18.

Table 1. Ochratoxin A concentrations in grape juice as detected by LC –MS/MS

Sample Name	Sample Type	Calculated Conc. (ng/ml)	Potential Ochratoxigenic Fungi
0.0 ng/ml	Blank	N/A	
0.30 ng/ml	Standard	0.30	
0.60 ng/ml	Standard	0.60	
Sample 1 J	Grape Juice	No peak (negative)	*A. niger aggreg., P. verrocosum*
Sample 2 J	Grape Juice	No peak (negative)	*A. niger aggreg.,*
Sample 3 J	Grape Juice	0.37	*Aspergillus niger aggreg., A. carbonarius, A. Sclerotium, A. ochraiceus*
Sample 4 J	Grape Juice	0.39	*Aspergillus niger aggreg., A. carbonarius, A. sclerotium, A. ochraceous ,P. verrocosum*
Sample 5 J	Grape Juice	1.85	*Aspergillus niger aggreg., A. carbonarius, A. Sclerotium, A. ochracieus, P. verrocosum,*



Figs 5-10. LC/MS-MS Chromatograms for 5-blank, 6-OTA standered 0,3ng/ml, 7-sample1 (negative), 8-sample2 (negative), 9-sample 3 (positive), 10-sample 5 (positive).

Results for the analysis of natural occurrence of OTA in five juice samples prepared from dried vine fruits as detected by LC-MS/MS is presented in Table 1. Out of five samples, three were found naturally contaminated with OTA (Figs 5-10). The three samples showed low levels of OTA contamination ranging between 0.3 ng/ml -1.58 ng/ml. The present result is in agreement with data obtained for natural occurrence of OTA in grape vine fruits harvested in Argentina [(14)] and also agree with those obtained by [(26)] who studied the presence of OTA on dried vine fruits samples from Lebanon. In grape juice, the maximum limit of 2ng/ml. was adopted in European Union countries (European Commission, 2006) [(27)]. The safe tolerance intake of 16 ng/kg of body weight per day was established by the joint expert committee on food additives of the world health organization (JECFA, 2007) [(28)]. The presence of this toxin in grape juice is of great concern for consumer's health.

It is interesting to note that the majority of fungi associated with naturally contaminated samples are potentially ochratoxin A- producing species [4, 12, 22, 25]. These include *A .carbonarius, A. niger* aggreg. (i.e.*A.niger* and *A.tubingensis*)., *A .sclerotium. A. ochraceus,* and *Penicillium verrucosum.* Among these, *A. carbonarius* was observed in several studies as the main source of OTA contamination in dried vine fruits [3, 12].

CONCLUSIONS

This study was provided information on fungal species responsible for contamination of dried vine fruits as well as the species associated with the naturally contaminated grape juice samples with ochratoxin A. The analyses of natural occurrence of ochratoxin a in juice samples prepared from dried vine fruits, showed 60% of juice sample contaminated with ochratoxin A levels ranging between 0.37-1.85 ng/ml as detected using L C-M S /M S technique. None of these positive samples exceeded the regulation set as suggested by EU.

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