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# Identification and Determination of Aflatoxin G1 and aflatoxigenic *Aspergillus* isolates from dried vine fruits in Duhok by LC/MS-MS technique

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**Abstract**

The study was undertaken to investigate the mycobiota of the 20 samples of dried vine fruits (Zibib) used for preparation of grape juice collected from local shops for soft drinks and fruit juices during August, 2010 and for natural contamination with aflatoxin G1.Aflatoxigenic strains of *Aspergillus* were detected by culture based methods , these include fluorescence upon exposure to UV long wave length (365 nm) light and pigment production in coconut agar medium after exposure to ammonium hydroxide . All tested of *A.parasiticus* showed aflatoxigenic potential and the ratio of aflatoxigenic isolates of *A.flavus* was higher than non aflatoxigenic strains. Natural contamination of grape juice with afflatoxin G1 was detected by LC/MS-MS technique. Out of 14 juice samples 7 were be found to contaminated with AFG1.

**Key words**: *aflatoxin G1, grape juice, LC/MS-MS.*

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## 1. Introduction

Aflatoxins (AFs) are fungal metabolites known for their potent carcinogenic properties .The ability of aflatoxin production has been reported in various species of the *Aspergillus* genus inside and outside the Flavi group [1]. However, various studies have indicated that aflatoxins are primarily produced as secondary metabolites by species of *Aspergillus*, particularly *Aspergillus flavus* and *Aspergillus parasiticus* [2] Additionally ,these studies indicated that the majority of *A. flavus* isolates (60-70%) are aflatoxigenic [1,3], where as almost all isolates of *A. parasiticus* are aflatoxigenic and are potential aflatoxin producers in agricultural commodities [4]. The most important members of AFs are AFB1, AFB2, AFG1, AFG2, and their two metabolites AFM1 and AFM2 contaminated foods, including corn, wheat, peanut, dried fruits and other crops resulting in illness or death of human and animal consumers [5]. The strain of *A. flavus* produce two aflatoxins AFB1, AFB2, but most strains of *A. parasiticus* produce all four toxins [6].

It has been well documented that not all strains are able to produce mycotoxin [7, 8] and hence this encouraged the use of

screening for their mycotoxin production potential.

We use highly sophisticated LC/MS-MS which give undoubtedly confirmed and quantitative results as well as qualitative analysis of wide range of molecules at trace levels. Several papers describing different kinds of MS methods for analysis of aflatoxins have been published [9-11].

The objectives of the present study were to identify the mycobiota associated with vine dry fruits (Zibib) collected from local markets and detect aflatoxigenic potential of isolates by rapid methods based on visual observations and determination of AFG1 contaminated grape juice prepared from Zebib by LC/MS-MS technique.

## 2. Materials and methods

### 2.1. Source of dried vine fruits (Zibib) samples:

Twenty dried vine fruit samples (used for preparation of grape vine juice were purchased from local shops (Duhok province) for soft drinks and fruit juice during August 2010 .The minimum sample size was 500 gram. Samples were stored in sterilized paper bags and stored in refrigerator at 5ºC. The samples were processed within one week after collection.

### 2.2. Grape juice preparation:

The grape juice was prepared as described [12]. The dried vine fruit (50g) were blended at high speed with four parts sterilized distilled water (250ml) in a commercial blender or home food processor for 10 min, with 1min rest each 3 min, to prevent sample heating. After that 3 ml of the juice were taken after passing through Millipore filter (0.22) µm and mixed with 7 ml methanol placed in sterilized Eppindrof vial and stored in refrigerator for aflatoxin detection.

### 2.3. Standard preparation:

The standard AFG1 was obtained from Sigma Aldrich Japan (Tokyo, Japan) was dissolved in acetonitrile at 1mg/ml and was stored at 4°C in the dark until use .To prepare the working standard for LC/MS-MS analysis the AF stock solution was equally pipetted and transferred to vial, and it was then diluted with mobile phase . The final concentration of aflatoxin G1 was 1ug/ml.

### 2.4. Isolation of fungi from dried grape samples:-

Dichloran 18% Glycerol Agar (DG18) medium (Hocking and Pitt, 1980) for xerophylic fungi were used for the isolation and enumeration of fungi from dried fruits. The medium was supplemented with Chloramphenicol (50 mg/L). Thirty dried vine fruits taken randomly from each sample, were treated with 2% sodium hypochlorite solution for 2 min. then rinsed in sterile water and dried on sterilized blotters. Raisins were aseptically placed on DG18 plates (10 per plate). All plates were incubated in darkness for 7 days at 25 ºC.

### 2.5. Identification of fungi

For the identification of species of the genus *Aspergillus*, pure colonies were grown on three media according to Klich [13].The media used were as follows: Czapeck Yeast Extract Agar incubated for seven days at 25˚C (CYA25), Czapeck Yeast Extract Agar incubated for seven days at 37˚C (CYA37), Czapeck Yeast Extract Agar with 20% Sucrose incubated for seven days at 25˚C (CY20S), Malt Extract Agar (MEA) incubated for seven days at 25˚C. Another confirmatory test was made by measuring growth abilities on Creatine Sucrose Agar (CREA) medium. Production of acid turning of the medium from purple to yellow can be used as a diagnostic feature for some *Aspergillus* species [14]. Ingredients and preparation of the above four media were mentioned [13, 15]. Each medium was supplemented with 50mg/L chloramphenicol (SDI) to suppress bacterial growth. For each culture, four plates were

used, two of CYA, one of CY20S and one of MEA. Each plate is inoculated with mycelial disc of each species (3 mm. diameter) at the center and incubated in the dark for seven days. One CYA plate is incubated at 37˚C. The rest are incubated at 25˚C. All inoculated plates were done in triplicate. All species identifications were according to the keys and descriptions provided [14, 16, 17].

**2.6. Determination of toxigenic potential of fungi in culture media:**

All identified *Aspergillus* isolates belong to section *Flavi* were tested for their aflatoxigenic potential. A rapid method for identification of aflatoxigenic strains of *Aspergillus* section *Flavi* depending on the color change after exposure to ammonia vapor were adopted as described [18]. Tested strains of the section Flavi were grown in Petri plates of coconut cream agar. The medium was prepared according to [19, 20]. Each strain was inoculated at the centre of solidified coconut cream agar medium in 9-cm diam. Petri dishes and incubated at 27C˚ in the dark. To observe the color change of colony reverse after 4 days incubation, dishes were placed upside down and a drop of ammonia solution was put into the lid of the Petri dishes.The colony reverse of the aflatoxin–producing strain turned pink [18]. Exposure of plates with aflatoxigenic strains to UV light (365nm) showed bright blue or blue-green fluorescent zone surrounding colonies [21].

**2.7. Determination of Aflatoxin G1 using High–Performance Liquid Chromatography–Tandem Mass Spectrometry LC-MS/MS:**

This was done at Princess Haya Biotechnology Centre. The University of Science and Technology Jordan during July 2010. The method of [11] was adopted. Chromatographic separation and MS Detection were performed using an Agilent 1200 Rapid Resolution LC and a 6460 Triple Quadruple Mass Spectrometer. The samples were directly injected and analyzed without further sample preparation.

For the LC method 5mMoL ammonium acetate (pH: 3.2) and methanol were used as mobile phases in gradient mode. The column (ACE 5 C 18 (100*2.1 mm) was kept at 55°C with a flow-rate of 0.4 ml/min. The total analysis time was set to 25 min. An ESI source with Agilent Jet Stream technology was coupled to the mass spectrometer.

Determination of the optimal MRM transitions for all analytes were carried out by flow injection analysis of standards at concentration levels around 0.1 ng/ml using Mass Optimizer, an automated MRM Method Development Software.

**3. Results and Discussion**

The genera and species of fungi isolated from dried vine fruits by direct plating method on DG18 medium and without surface disinfection and their frequency of occurrence are presented (Tables 1, 2)

A total of 43 species assigned to 18 genera in addition to Yeasts and non–sporulating mycelia were identified. *Aspergillus* showed the wide spectrum (18), followed by *Penicillium* (8 species) , *Cladosporium* (2), *Emericella* (2), *Rhizopus* (2) , whereas the remaining genera were represented by one species each. The predominance of *Aspergillus* in dried vine fruits was expected because members of this genus can survive drying process due to relative resistance of their spores to sunlight and UV radiation, in addition to their ability of production of sclerotial propagules [16, 22]. *Eurotium* and *Monoascus* species can grow exceptionally well at low water activity. They are therefore common in foods with high concentrations of sugars [15].

Within *Penicillium*, there are resistant species capable of growing at low water activity and tolerating high temperature [23]. Other relatively frequent contaminants were *Alternaria*, *Absidia*,*Aureobasidium*

*Trichoderma*, non sporulating mycelia and yeast. Similar result was found [22].
*Aspergillus niger*, *A.carbonarius* and *A.flavus* have been found as the most frequent isolates from dried vine fruits with a percentage of occurrence of 90% and 61.66% and 18.8%, respectively (Table 2).

In Argentina six species of *Aspergillus* section *Nigri* were detected from samples of dried vine fruits while the predominant species were *A.niger*, *A.awamori*, *A.carbonarius*[24].

**Table 1. % occurrence of the fungal genera isolated from dried fruits on DG18.**

| No | Fungal genus | % Occurrence on DG18 |
|---|---|---|
| 1 | *Absidia* | 5 |
| 2 | *Alternaria* | 40 |
| 3 | *Aspergillus* | 90 |
| 4 | *Chaetomium* | 10 |
| 5 | *Cladosporium* | 20 |
| 6 | *Emericella* | 13 |
| 7 | *Eurotium* | 70 |
| 8 | *Fusarium* | 20 |
| 9 | *Monilia* | 5 |
| 10 | *Monoascus* | 6 |
| 11 | *Mucor* | 10 |
| 12 | *Paecilomyces* | 7 |
| 13 | *Penicillium* | 75 |
| 14 | *Phaeoacremonium* | 5 |
| 15 | *Rhizopus* | 36 |
| 16 | *Scytalidium* | 5 |
| 17 | *Sterile mycelium* | 5 |
| 18 | *Trichoderma* | 20 |
| 19 | *Stachybotrys* | 5 |
| 20 | *Yeast* | 15 |

**Table (2). Occurrence (%) of fungi isolated from dried vine fruits and their frequency of occurrence.**

| No. | Fungi | % Occurrence on DG18 |
|---|---|---|
| 1 | *Aspergillus .aculeatus* Iizuka | 8.83 |
| 2 | *A.awamori* Nakaz | 10 |
| 3 | *A.candidus* Link | 5.3 |
| 4 | *A.carbonarius* (Bainier) Thom | 61.6 |
| 5 | *A.flavus* Link | 18.83 |
| 6 | *A.carneus (Tiegh.) Blochwitz* | 2.1 |
| 7 | *A.caspitosus* | 1.3 |
| 8 | *A.flavipes* Bainier & Sartory | 1.67 |
| 9 | *A.foetidus* Thom & Raper | 6.17 |
| 10 | *A.fumigatus* Fresen | 10 |
| 11 | *A.niger* Tiegh.*nom.cons.* | 90 |
| 12 | *A.ochraceus* K.Wilh | 2.3 |
| 13 | *A.oryzae (Ahlburg.) Cohn* | 3.4 |
| 14 | *A.ostinus* | 3.2 |
| 15 | *A.parasiticus* Speare | 10 |
| 16 | *A.restrictus* G. Smith | 3.3 |
| 17 | *A.terreus* Thom | 10 |
| 18 | *A. tamarii* | 2.5 |
| 19 | *P.aurantiogresum* Dierckx | 2.5 |
| 20 | *P.brevicompactum* Dierckx | 7 |
| 21 | *P. citrinum* Thom | 10.3 |
| 22 | *P.chrysogenum* Thom | 3 |
| 23 | *P.expansum* Link | 12 |
| 24 | *P.glabrum* (Wehmer) Westling | 8.5 |
| 25 | *P.spinulosum* | 2.17 |

| No. | Fungi | % Occurrence on DG18 |
|---|---|---|
| 26 | *P.verrocosum* Dierckx | 3 |
| 27 | *Alternaria alternate* (Fr.) Keissl | 7.3 |
| 28 | *Cladosporium cladosporoides* (Fresen.) G.A. de Vries | 12 |
| 29 | *Cladosporium herborum* (Pers.) Link | 1.6 |
| 30 | *Emericella nidulans* (Eidam) Vuill | 10 |
| 31 | *E.quadrilineata* Thom & Raper | 10 |
| 32 | *Eurotium herboirorum* Link | 10 |
| 33 | *Fusarium oxysporum* Schlecht | 5 |
| 34 | *Monoascus sp.* Tiegh. | 1.6 |
| 35 | *Monilia (*Pres*)* | 10 |
| 36 | *Mucor circinelloides* Tiegh | 13.3 |
| 37 | *Paecilomyces varioti (*Bainier*)* | 4 |
| 38 | *Rhizopus solani* Ehrenb. | 10 |
| 39 | *R.stolonifer* (Ehrenb.) Vuill. | 10 |
| 40 | *Sterile mycelium (white)* | 3 |
| 41 | *Scytalidium* sp. | 5 |
| 42 | *Stachybotrys* sp. | 1.2 |
| 43 | *Trichoderma* sp. | 1.3 |
| 44 | *Ulocladium atrum* Preuss. | 6 |
| 45 | *Yeasts* (pink) | 3.5 |

### 3.1. Aflatoxigenic abilities of isolates of *Aspergillus* section Flavi on culture based methods.

Table (3) shows the results of screening *Aspergillus* section Flavi isolates for aflatoxigenic production abilities. Out of 113 isolates of *A.flavus*, 91 isolates (80%) were positive. However, in a recent study [25] showed that 62.5% of *A.flavus* from corn and 55.5% from sunflower seeds displayed positive aflatoxigenic ability, while, Mohammed *et al*. [21] showed 81.8 % of isolates of *A.flavus* isolated from different agricultural commodities had aflatoxigenic ability. Table (3) shows that all strains of *A.parasiticus* isolates showed positive results (100%). This result is in line with previous reports on the aflatoxigenic potential of *A.parasiticus* strains isolated in Iraq [25].

**Table 3. The ability of some isolates of *Aspergillus* section *Flavi* isolated from dried vine fruit to produce Aflatoxin *in vitro*.**

| Fungal isolates | No. of isolates | Positive isolates | % of positive isolates |
|---|---|---|---|
| *A. flavus* | 113 | 91 | 80 % |
| *A. parasiticus* | 80 | 80 | 100 % |
| *A. oryzae* | 25 | 0 | 0 |
| *A. tamarii* | 18 | 0 | 0 |

### 3.2. Detection of AFG1 naturally contaminated dried vine fruit by LC/MS-MS.

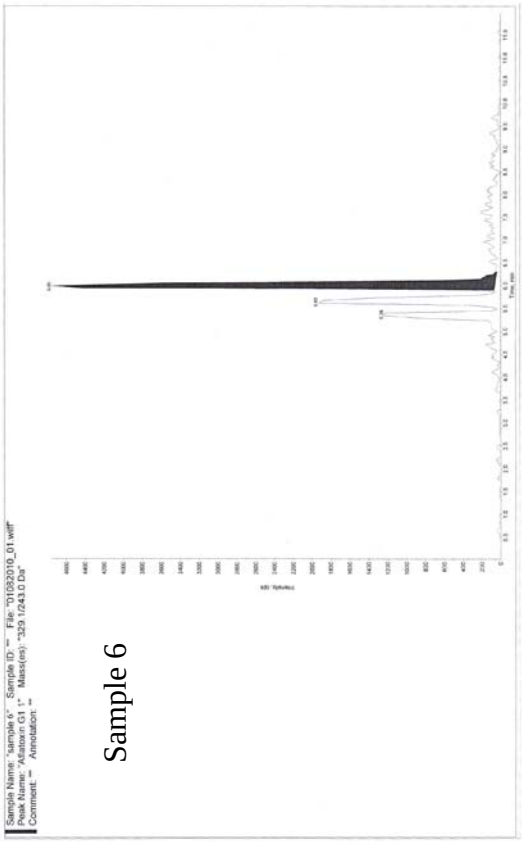
Seven samples out of 14 (50%) samples of grape juice samples were contaminated with AFG (Table 4, Fig. 1). Samples contaminated with *A. flavus* and *A .parasiticus* showed the highest contamination level, whereas samples with *A. oryzae* showed negative results, these results are similar with other studies [27-29].

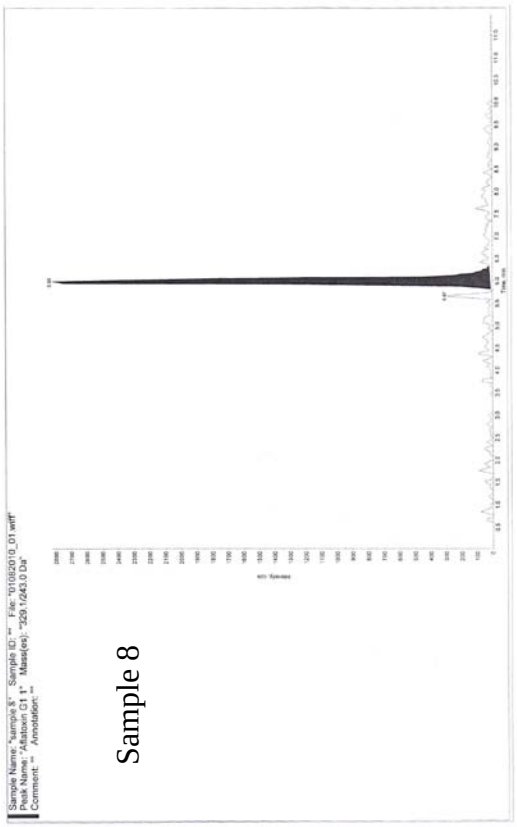
It is interesting to note that several samples of dried vine fruits were contaminated with *Aspergillus* section Flavi and particularly *A.parasiticus*. The later species was observed in the other study as the main source of AFG1 contamination in dried vine fruits [16].

**Table (4). Positive and negative samples contaminated with AFG1 and their associated fungi**

| Sample name | Potential Activity | Fungi Associated with samples |
|---|---|---|
| Sample 1 | -ve | *Aspergillus tamarii, A. carbonarius, Penicillium verrucosum* |
| Sample 2 | -ve | *A.aculeatus, A.candidus, A.flavipes ,P.expansum* |
| Sample 3 | -ve | *A.carneus, A.fumigatus, A.japonicus, P.glabrum* |
| Sample 4 | -ve | *A.niger ,A.caspitosus, A.foetidus, P.glabrum, A.oryzae* |
| Sample 5 | +ve | *A.flavus, A.niger, Aniveus, A.ostinus, P.spinolusum* |
| Sample 6 | +ve | *A.parasiticus, A.oryzae, P.citrinum, P.glabrum* |
| Sample 7 | -ve | *A.niger, A.oryzae, P.aurantiogresum* |
| Sample 8 | +ve | *A.flavus, A.oryzae, P.expansum, A.aculeatus* |
| Sample 9 | +ve | *A.flavus, A.oryzae, A.niger, A.tamarii,P.brevicompactum* |
| Sample 10 | -ve | *A.niger, A.foetidus* |
| Sample 11 | +ve | *A.parasiticus, P.expansum, A.foetidus* |
| Sample 12 | +ve | *A.flavus, A.tamarii, P.citrinum* |
| Sample 13 | +ve | *A.parasiticus, A.niger, A.awamorui* |
| Sample 14 | -ve | *A.niger, A.oryzae, A. tamarii* |

**(+ve) positive, (-ve) negative**



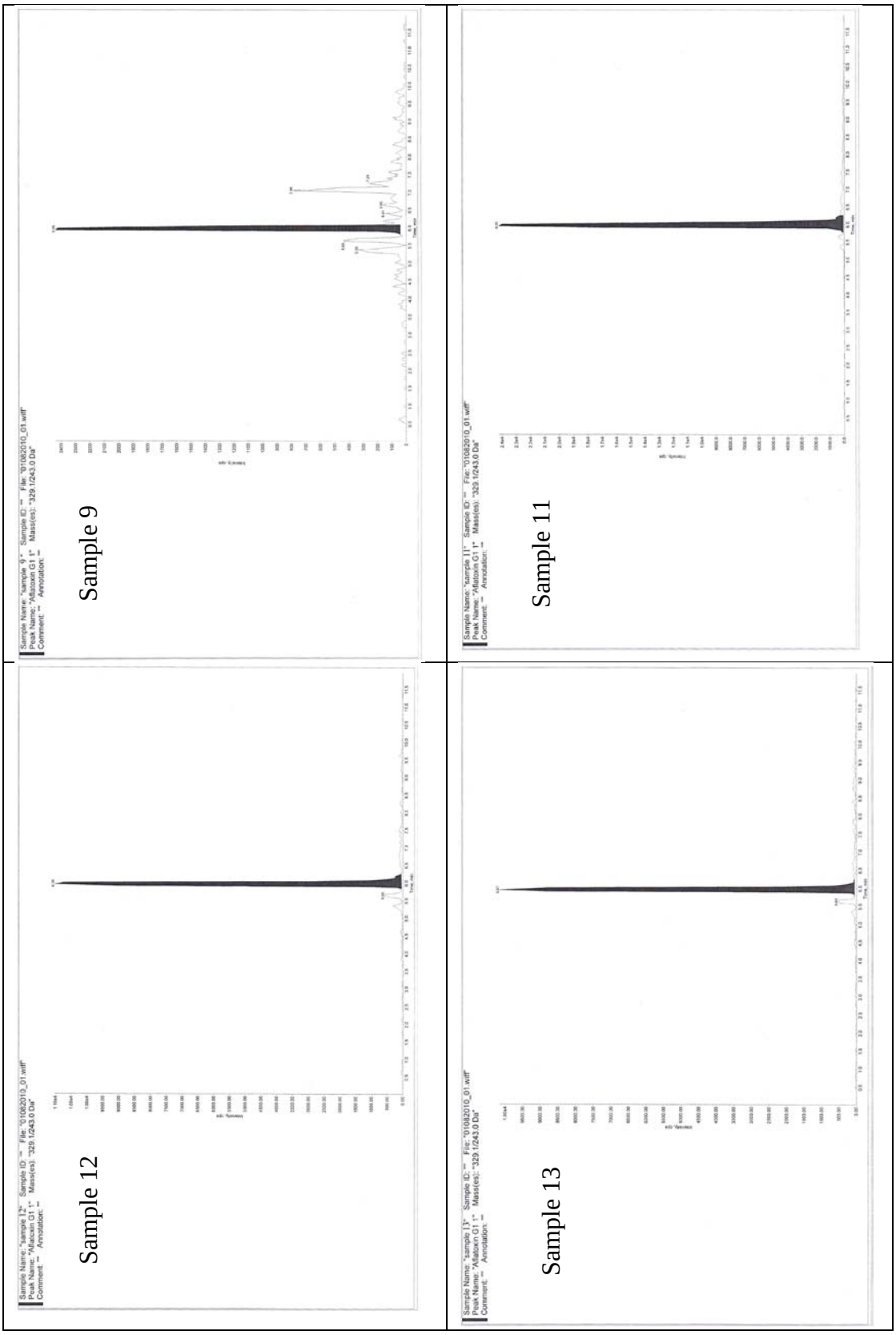



**Figure 1. Aflatoxin G1 detected from grape vine juice by LC/MS-MS**

The present work indicated that dried vine fruit examined were contaminated with several fungi, many of these fungi are capable of producing aflatoxins .These findings indicate that there may be a risk of human exposure to aflatoxin through the consumption of dried vine and grape juice. Thus such contaminated materials should be monitored before used.

**4. Acknowledgment:**

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**الخلاصة**

هدفت هذه الدراسة للتحري عن تواجد الفطريات المصاحبة لثمار الأعناب الجافة (الزبيب) والتي تم الحصول عليها من أسواق ومحلات بيع العصائر في محافظة دهوك خلال شهر أب 2010 وتلوثها بالسم الفطري أفلاتوكسين. تم تشخيص الأنواع المنتجة لسم الأفلاتوكسين باستخدام تقنيات تعرض الوسط الزرعي إلى أشعة UV وإنتاج الأصباغ بعد تعرض الوسط لبخار الأمونيا. أوضحت النتائج إن نسبة عزلات الأنواع المنتجة لسم الأفلاتوكسين في الفطر *A. flavus* أعلى من العزلات الغير المنتجة. بينما أعطت جميع عزلات الفطر *A.parasiticus* قابلية لإنتاج الأفلاتوكسين . تم استخدام تقنية LC/MS–MS للكشف عن السم الفطري أفلاتوكسين G1 تم اختبار 14 عينة من العصير عشوائياً وجدت 7 عينات ملوثة بالسم الفطري AFG1.