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OCHRATOXIN A RESIDUE IN GRAPE JUICE CONCENTRATE

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ABSTRACT

Grape juice concentrate (GJC) is evaporated, concentrated and shelf-life extended form of grape syrup. Since ancient times, evaporated grape syrup has been traditionally produced in most Iranian regions by using varieties of grapes. As GJC is generally produced from the year-end harvest of poor quality, ochratoxin has been a concern in this product**.** As there has been no research to evaluate the OTA in GJC so the aim of this study was to investigate this mycotoxin in GJC samples in Khorasan province in north eastern of Iran. For this purpose, 20 GJC samples were collected from retail stores of 9 cities in Khorasan province. Samples were analyzed for OTA content by high performance liquid chromatography (HPLC) technique. Results showed that 12 GJC samples (60%) contained detectable amounts of OTA by average concentration of 0.6±0.71 ppb which is lower than EU standards (2 ppb). The highest and lowest concentration of OTA in the samples was 0.24 and 1.74 ppb, so OTA poisoning has not been a concern in the GJC samples.

Keywords: Grape juice concentrate, Ochratoxin, HPLC, Food safety.

INTRODUCTION

Mycotoxins are secondary metabolites of molds, which are associated with certain disorders in animals and humans. In addition to being acutely toxic, some mycotoxins are now linked with the incidence of certain types of cancer, and it is this aspect that has evoked global concern over feed and food safety [1]. Mycotoxins such as aflatoxins (AFs) and ochratoxin A (OTA) can grow easily according to the unsuitable conditions of growth, harvest, transport, and storage [2]. Ochratoxin has received more attention because, it is suspected of causing cancer of the urinary tract and damage to kidneys [3].

OTA is a mycotoxin produced by several fungal species from *Aspergillus* and *Penicillium* genera. It has been found to

occur naturally in plant products such as beans, cereals, cocoa, coffee, dried fruits, grapes, pulses, soybean and spices, and also in their industrial derivatives worldwide [4, 5, 6, 7].

According to the codex alimentarius standard [8] and also the EU regulation [9] the maximum level of OTA in dried vine fruit (currants, raisins and sultanas) should not exceed 5ppb, and the maximum limit for grape juice, concentrated grape juice as reconstituted, grape nectar, grape must and concentrated grape must as reconstituted is 2 ppb. According to Iranian national standard the maximum acceptable amount of OTA in fig, date and raisin is 10 ppb [10] but no limit is set for OTA in fruit beverages and nectars.

Different studies have been done to detect OTA in foods such as in cocoa beans [11], wheat [12], maize bread [13], pepper [14]. Several reports revealed the presence of OTA in grapes as consequence of contamination with *Aspergillus ochraceus* and *Penicillium verrucosum* [15, 16, 17, 18]. OTA was detected in red grape-juices for the first time with an average concentration of 0.235 ppb. White grape-juices as well as other fruit juices (two apple, six orange, two others) did not contain OA (<5000 ppb) [19]. But there has been no study on the detection of OTA in GJC. So the objective of this study was to evaluate the occurrence of OTA using high performance liquid chromatography (HPLC) technique in GJC distributed in Khorasan province in north east of Iran.

MATERIALS AND METHODS

Samples Traditionally produced GJC samples were used as material. Twenty GJC samples were collected during spring 2012 from retail stores of 9 cities including Mashad, Kashmar, Torbat-e-heydarie, Qaaen, Birjand, Bojnourd, Gonabad, Qouchan and Sabzewar in Khorasan province in Iran. All the samples had been produced between sept 2011 and may 2012 and All the regions which samples were taken from, had similar cilmates.

Chemicals

All used chemicals were analytical or HPLC purity grade: sodium chloride, phosphate buffered saline (PBS), glacial acetic acid, toluene 99%; acetonitrile and methanol; polyethyleneglycol 6000 (Merck, Hohenbrunn, Germany); ochratoxin A 99% (Sigma-Aldrich Chemie, Steinheim, Germany); immunoaffinity columns Ochraprep (R-Biopharm Rhone, Glasgow, Scotland).

Methods and analysis

Preparation of standard OTA solution

The stock OTA standard solution was prepared by dissolving 5 mg crystalline OTA in 4 ml of the mixture toluene-acetic acid 99:1 (v/v). This solution was stored at -

18°C. The working standard solutions for calibration and spiking purposes were prepared step by step by vacuum evaporating of necessary stock solution volume, dissolving of the residue in the mobile phase and sequential diluting of this solution. The working standards were stored at 4 °C.

GJC clean-up on immunoaffinity column (IAC) Immunoaffinity columns Ochraprep for OTA isolation from GJC were employed. Immunoaffinity columns commonly stored at 4°C were kept in ambient temperaqture before use. The fill in IAC column was then conditioned with the filling solution present in the IAC column. 2 g of sample diluted (2:23, w/w) with the dilution solution was then applied on the column and let pass through the column without or with applying a slight vacuum. IAC was then washed with 20 ml of washing solution to get rid of interfering substances. The column was then dried with air for 10-15 seconds and the retained OTA was eluted with 1500µl of methanol-acetic acid (98:2 v/v). The obtained eluate was evaporated on a rotary vacuum evaporator to dryness. The residue was dissolved in 0.25 ml of the mobile phase and quantitatively transferred into 2 ml dark sampler vial.

Apparatus The HPLC equipment Agilent Technologies 1100 Series (Halbron, Germany) with auto-sampler and fluorescence detector at excitation wavelength 333 nm and emission wavelength 460 nm was used. The analytical column Zorbax SB-C18, 250 × 4.6 mm with the sorbent particle size of 5 µm together with the pre-column Zorbax SB-C18, 12.5 × 4.6 mm with the same particle size (Agilent Technologies, Halbron, Germany) was applied. The mobile phase mixed of methanol: H2O: acetic acid (29.3:69.7:1) has flown through the system at the rate of 1 ml.min-1. Samples were injected onto analytical column in 100 µl volume. All analyses were carried out at ambient temperature.

Evaluation of the method The analytical procedure was internally validated by means of calibration curve and recovery test. The calibration measurements were carried out with OTA standard solutions at concentrations 0.5, 1, 2, 2.5, 5, 10 and 15 ppb. The recoveries of OTA using IAC columns for sample pretreatment were studied by spiking GJC samples with standard solutions at OTA levels of 2 and 5 ppb.

Statistical analysis

The results were analyzed by Excell 2007 software and results expressed as X±SD.

RESULTS AND DISCUSSION

Ochratoxin occurrence in investigated samples The standard solutions of

concentrations from 0.5 to 15 ppb OTA were used to find calibration/standard curve as described by the following regression equation:

$Y=1.803*X+0.176$ (1)

where y=area and x=amount of OTA. The results showed the linearity of the standard curve over the range studied. The coefficient of determination (R^2) was 0.99653. Figure 1 gives the calibration curve of standard solutions of OTA with the concentrations of 0.5, 1, 2, 2.5, 5, 10 and 15 ppb by HPLC analysis.

The levels of OTA contamination in GJC samples examined in our study are shown in Table 1. Twelve (60%) out of 20 samples were found to contain OTA levels ranging from 0.24 to 1.74 ppb. Most importantly, none of the samples exceeded the maximum tolerable limit for OTA as stated in the codex alimentarius standard and also the EU regulation (2 ppb).

These amounts are considered to be much lower than the limit value of 3 ppm as defined in the Turkish Food Codex [20] for pekmez (GJC) and consuming condition has no risk for human health. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established the provisional weekly intake of OTA to 120 ng/kg of body weight.

There has been conducted few studies on OTA determination in grape products and no research on GJC. Belajova et al, (2007) analyzed **t**hirty nine samples of white and red variety wines from the Slovak wine production of 2005 as well as 16 samples of imported wines OTA by HPLC method [21]. OTA was not detected in more than 50% of wine samples. The level of OTA in red wines was a little higher than in white ones (the highest concentration found was 0.463 ppm). Arici et al. (2004) studied the fate of ochratoxin A during the pekmez production from mouldy grapes [22]. The grape juices produced from mouldy grapes, contaminated naturally with OA between 2.1 and 9.8 ppb were used in pekmez production. In the processing steps of pekmez, changes in OA amount were examined. The amounts of OA in pekmez samples were found to be 5–6 times higher than OA amount of grape juice.

Fredj et al, (2009) conducted a survey to access mycotoxin-producing fungi and to evaluate the ochratoxin A (OTA) and the Aflatoxin B1 (AFB1) potential production for fungal strains contaminating table grapes in different Tunisian vineyards [23]. Among 100 *Aspergillus* isolates, *Aspergillus niger* aggregate were the most frequent (70%) followed by *Aspergillus carbonarius* (7%) and *Aspergillus flavus* (23%). The mycotoxigenic capacity of the isolates was tested in culture media revealed that the highest levels of OTA production were

obtained with strains of *A. carbonarius* (80% of them) whereas only 5% of *A. niger* aggregate were OTA producers. Also Rossi et al, (2011) mentioned to *Aspergillus niger and Aspergillus carbonarius* as the main OTA producers in wine grape [24] while Benkhemmar et al, (1993) indicated to *Penicillium* species to be the main genus in mouldy grapes which are capable to produce OTA [25]. The possibility of development of moulds producing OTA depends on climatic conditions, though it is more frequent in areas with tropical climate [19]. The favourable factors for the production of mycotoxin comprise suitable temperature, moisture, aeration, and duration of incubation and interaction of fungi [26].

Generally, in rural regions, mouldy grapes are used in GJC production and the proceess is conducted in poor sanitation conditions. This article was the first study on this subject in Iran. It is suggested that OTA content should carefully be considered in the GJC production. The prevention of grape contamination with OTA in whole reposes by applying the basic sanitary measures is recommended.

CONCLUSION

This limited study is the first information on OTA presence in GJC in Iran. Moreover, the analytical method presented and used in this survey, has shown sufficient parameters and sensitivity for detecting traceable OTA levels in GJC. In general, GJC produced and sold in Iran had lower level of OTA than the proposed European limit of 2 ppb. However it should be emphasized that the presence of OTA in grape is strongly dependent on climatic conditions during the maturation and harvest of grape and storage conditions after harvesting. On the other hand sanitary requirements have to be adhered specially at storage to produce GJC not contaminated or minimally contaminated with OTA.

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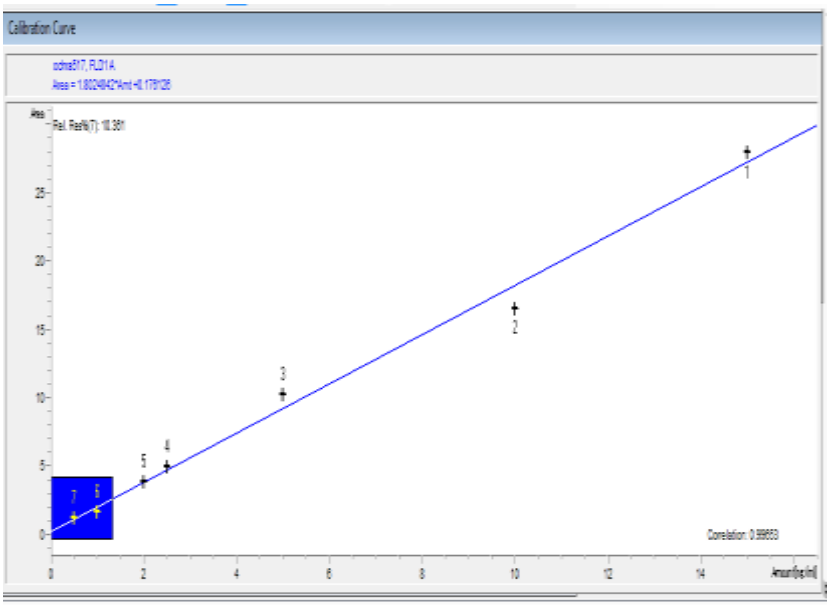

Figure 1: Calibration curve of standard solutions of OTA with concentrations of 0.5, 1, 2, 2.5, 5, 10 and 15 ppb by high-performance liquid chromatography analysis

Table1. Levels of OTA (ppb) in GJC samples

Region	No. of samples	OTA concentration (ppb)
Mashad	4	1.74
Kashmar	2	0.98
Torbat-e-heydarie	2	0.9
Qaaen	2	1.58
Birjand	2	nd*
Bojnourd	2	nd
Gonabad	2	nd
Qouchan	2	0.24
Sabzewar	2	nd
Sum	20	0.6±0.71

*Not detectable