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The Production Pattern of Aflatoxin G₁, G₂, by HPLC

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ABSTRACT

Objective: Aflatoxin G₁ and G₂ are highly toxic and carcinogenic secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* fungi sampling was done according to "CBS" instructions from indoor and outdoor stations. **Methods:** In HPLC measuring, we first purified our sample of all Aflatoxins, We used imonoaffinty columns, Finally the device compared average of the results of the samples curve areas with average of the results of standard curve areas and showed the effective material value based on ppb. Geographical distribution of 107 mentioned samples and The frequency of subgenuses in different regions, The minimum, average and maximum amount of toxins, The average amount of produced toxin in culture medium were determined. **Results:** In comparison, the average of aflatoxin G₁ and G₂ with HPLC method in biomass the highest examined amount is in the range of -0/5 -0/5 ppb which is produced by fungi such as *A. niger*, *A. flavus*, *A. fumigatus*. In biomass, the correlation between aflatoxin G₁ and G₂ measured by HPLC method isn't any reverse relation and meaningful numeral differences. In culture medium, checking the average value of aflatoxin G₁ and G₂ which is measured by HPLC method, we can see a correlation and there is a meaningful numeral difference.

1. INTRODUCTION

Aflatoxins are toxic metabolites produced by different species of toxigenic fungi, called mycotoxins. Humans can be exposed to aflatoxins by the periodic consumption of contaminated food, contributing to an increase in nutritional deficiencies, immunosuppression and hepatocellular carcinoma. The order of the severity of acute and chronic toxicity is B₁ > G₁ > B₂ > G₂ reflecting the role of the 8,9 double bond and greater potency associated with the cyclopentanone ring of the series (Eaton & Gallagher, 1994). Human can be exposed to aflatoxin by the periodic consumption of contaminated food, contributing to an increase in nutritional deficiencies,

immunosuppression and hepatocellular carcinoma (Coppock & Christian, 2007). A lot of efforts, resources and time have been committed to Aflatoxin research work since the early 1960s (Windhom et al., 2002). Aflatoxin often occurs in crops in the field prior to harvest. Postharvest contamination can occur if crop drying is delayed or if water is allowed to exceed critical values during storage leading to mold growth (Craufurd P.Q et al., 2006). Aflatoxins are produced by members of *Aspergillus* and are common and widespread in nature (Gupta A ET AL., 2002). They can colonize and contaminate grain before harvest or during storage. Host crops are particularly susceptible to infection by *Aspergillus* following prolonged exposure to a high humidity environment or

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damage from stressful conditions such as drought, a condition which brewers the Barrier to entry (kankar A et al., 2005, Ayejuyoo .o et al., 2008).

Number publication describe analytical and cultural methods for the detection and quantification of aflatoxins in agricultural commodities and in cultures of fungi isolated from them. these methods vary in accuracy and precision, depending on the goal of the analysis. Analytical methods for aflatoxin detection and quantification (Nilufar and boyacioglu., 2002. Whitaker et al., 1996) Thin layer chromatography (TLC) (Stroka and Anklam., 2000) high-performance liquid chromatography (HPLC) (Seitz., 1975) Liquid chromatography mass spectroscopy (LC/MS) enzyme-linked immunosorbent assay (ELISA) (patay et al., 1989) and (v) immunoaffinity whit fluorescence (VICAM) (Nasir and Jolley., 2002).

2. MATERIALS AND METHODS

From the first days of May to the last days of October 2011, sampling was done according to "CBS" instructions from indoor and outdoor stations. One sample group was taken from among 50000 meter square area fields and also per processing plant using settle plates based on CBS rules too. Six plates including Malt extract agar (MEA), Yeast extract agar (YEA), Czapeks agar (CZA), Czapeks Yeast extract agar (CZYA), Saboruds dextrouse agar (SDA) and Potato dexterosus agar (PDA), all with 100 ppm chloramphenicol and 50 ppm tetracycline were applied for one sample group. Plates removed after 30, 60 and 90 minutes for outdoor and 15, 30 and 60 minutes for indoor sites, respectively. All the plates were incubated at $25 \pm 2^\circ\text{C}$ aerobically, then examined in the periods of 3, 7 and 15 days. We could identify any growing so that they were marked and harvested then cultivated in the prepared plates. Finally, for macroscopic and microscopic morphology examinations, 107 colonies were cultivated and grown at $25 \pm 2^\circ\text{C}$. In order to identify and rank the colonies, various methods were used. Front and back of one-two week colonies were selected for morphologic examinations and microscopic and macroscopic photography. Measurement of the colonies width, examination of front and back color of pigments, photography of mushroom umbrella, cells and grown masses, and also examination of creation and micrometry of asks were done by stereoscope. In all of the samples, micrometry and photography of conidiophores, wisicole and asks were done by micro analysis microscope.

2.1. HPLC measuring

In order to do HPLC process, we first purified our sample of all *Aflatoxins*. We used imonoaffinty columns. According to the test instructions, extraction of toxin was done by using methanol solution and water.

Initially, we injected extracts to the columns. The column includes gels containing antibodies specific for *Aflatoxin B and G* variants. At the next step, the column was washed by PBS solution. This leads to remaining *B and G Aflotoxin* which attached to gel and removal of other materials of cell extract. Then, the column was washed by special ethanol solution. The washing method was a two-phase process; at first phase, 1250 ml and then 500 ml of the solution was added to the column in such a way that whole of it was washed carefully. Finally, the attached toxin, was separated from the column using methanol and collected into particular vial.

2.2. Preparation and work with device

The mobile phase including one of water, methanol, acetonitrile, phosphate buffers or a mixture of them, was entered in the column and poured into the device bottle. Then the detector was turned on and set to 360 nm wave length and the electronic recorder switched on and speed of mobile phase current justified based on RP. Finally, the device was placed in the current, for 30 to 45 minutes without injection. After 45 minutes, absorption of the mobile phase was stopped, system suitability test was added and the results recorded. As a result, resolution factor, middle peaks, tailing factor, number of sub-pages of the column for measuring test material (theoretical plates), and capacity factor were calculated. After this step, the standard solution was injected 5 times to the device and its scale value was obtained. This value was bigger than %98. Then each test standard was injected 3 times and reference standard solution injected, afterwards. Finally the device compared average of the results of the samples curve areas with average of the results of standard curve areas and showed the effective material value based on ppb.

2.3. Statistical analysis

In this trial, we utilized k2 independence test and Anova table, which are used to show correlation or irrelolation of two classified variables (Bhat RV., 2003).

3. RESULTS AND DISCUSSION

Geographical distribution of 107 mentioned samples is as follows: 68 samples (%63.3) are from Eastern Gilan, 30 ones (%23) from Western *Gilan* and 9 ones (%8.4) from Western *Mazandaran*, with the least share of Genus *Aspergillus* samples. The frequency of subgenuses in different regions is as follows: *Circumdati* determined in 56 samples (%61.68) - the highest frequency, *Nidulantes* in 14 samples (%13.1), *Ornati* in 6 samples (%5.6) and 16 samples (%15) were grouped as unknown subgenus members as *A. sp I-VI*. Subgenus *Circumdati* including: *A. alliaceus* (n=2), *A. awamori* (n=3), *A. candidus* (n=4), *A. carbonarius* (n=6), *A. flavus* (n=17), *A. foetidus* (n=4), *A. melleas* (n=3), *A. niger* (n=4), *A. ochraceus* (n=4), *A. ostianus* (n=3), *A. parasiticus* (n=5), *A.*

soja (n=8), *A. wentii* (n=3). Subgenus species of *fumigati* include *A. fumigatus* (n=5), subgenus species of *Nidalantes* include *A. niveus* (n=3), *A. terreus* (n=7), *A. unguis* (n=4) and subgenus species of *ornate* include *S.ornata* (n=6) and unknown subgenus types including *A. Af flavus* (n=1), *A. af nidulans* (n=2), *A. sp III* (n=7), *A. sp IV*(n=2), *A. sp v*(n=2), *A. sp VI* (n=2).

The minimum, average and maximum amount of toxins in *circumdati* species in biomass extracts are as follows: for *A. ochraceus* 0, 17.94 and 40.75 ppb; for *A. ostianus* 39.20, 41.53 and 48.42 ppb; for *A. melleus* 33.22, 39.12 and 44.48 ppb; for *A. candidus* 30.92, 36.12 and 42.52 ppb; for *A. flavus* 0, 22.46 and 49.506 ppb; for *A. sojae* 0, 21.61 and 55.06 ppb; for *A. parasticus* 26.79, 30.83 and 35.06 ppb; for *A. allaceus* 0, 14.66 and 29.32 ppb; for *A. awamori* 0, 9.11 and 27.35 ppb; for *A. carbonarius* 0, 6.35 and 38.11 ppb, respectively. The minimum, average and maximum amount of toxins in subgenus *Nidulnates* is as follows: 0, 23.75 and 47.50 ppb, respectively. In *Ornata* subgenus *ornati* minimum, average and maximum were 0, 28.17, 45.62 ppb, respectively. Also for *Fumigati*, *A. fumigatus*, minimum, average and maximum of toxin was 0, 22.15 and 41.69 ppb. Finally in unknown sample (sp III) the minimum of produced toxin was 38.16 ppb, its average amount was 39.55 ppb and its maximum amount was 39.95 ppb. The average amount of produced toxin in culture medium was as follows: in subgenus *Circumdati*, *A. foetidus* 2.58 ppb; in *A. mellus* and *A. ochraceus*, the 0; in *O.ostianus*, 2.74 ppb; in *A. cantidus* 0.3 ppb and in *A. flavus* 0.45 ppb. About subgenus *A. wentii*, the average of toxin was 0; in *A. sojen* 0.39 ppb; in *A. parasiticus* 0.29 ppb; in *A. alliaceus* 0.49 ppb; in *A. niger* and 0.22 ppb; in *A. awamori* 0.25 ppb and in *A. carbonarius* 90.28. The average amount of produced toxin in *Oranati*, subgenus *S.ornati* was 0; in *fomigati* 0.6 ppb; in *nidulantes* was 1.31 ppb and in unknown genus from 0.40 ppb to 0.92 ppb.

In comparison, the average of aflatoxin G_1 with HPLC method in biomass the highest examined amount is in the range of 0-5 ppb which is produced by fungi such as *A. niger*, *A. flavus*, *A.candidus* the average ,maximum and minimum of aflatoxin G_1 with HPLC method is to 0. 0 and average value of aflatoxin G_1 calculated by HPLC method in culture medium, was in in the range of 0-1 ppb that has been seen by fungi such as *A. niger*, *A. flavus*, *A. fumigatus* and *A.candidus*. . The minimum value of Aflatoxin G_1 was more than 2 ppb and the average of its maximum is less than 5 ppb. According the average of aflatoxin G_2 with HPLC method in biomass the highest examined amount is in the range of 0-5-0/5ppb which is produced by fungi such as *A. niger*, *A.flavus* .also the average ,maximum and mimimum of aflatoxin G_2 with HPLC method is to 0 and the average of aflatoxin G_2 with HPLC method in culture medium the highest examined amount is in the range of -1 - 1 ppb which is produced by fungi such as *A.niger*. the average

,maximum and minimum of aflatoxin G_2 with HPLC method is to.

In biomass, the correlation between G_1 and G_2 Aflatoxin measured by HPLC method average, there isn't any reverse relation and meaningful numeral differences. In culture medium the average of aflatoxin of G_1 and G_2 which is measured by HPLC, we can see a correlation and there is a meaningful numeral difference (Sig: 0.000, pc: 0.930). According to the fact that numeral difference resulted from toxin measurement in statistical examinations has been meaningful, so we can find out that except the confirmed correlations and non alignment between them, the numeral difference regarding to error tolerance of commercial kits is reasonable and this led to the meaningful difference. In examining meaningfulness of the numeral difference and the kind of correlation between average values of whole Aflatoxin G_1 and G_2 each one separately measured by HPLC, no meaningful numeral difference between the cases was seen and also no significant correlation despite reverse relation, we can point to above cases (Khurgami et al .,2012,Vahidi et al ., 2012, Mehdikar et al., 2011) .

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