The Mycotoxin contamination of the imported consumer rice and its producing fungi in Zabol

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Abstract

Introduction:
Mycotoxins leave severe harmful effects on human beings’ and animals’ organs and huge economic damages. Different Mycotoxin contamination in cereals, especially in rice, is an important issue in the field of food hygiene and public health of the society. Due to the particular position of rice in the food basket of Iranian households and the high consumption of rice and its derivatives, the present study was conducted to examine the mycotoxin contamination of the imported consumer rice and its producing fungi in Zabol City.

Materials and Methods:
In this research, 123 rice samples were collected using a non-probability convenience sampling method. After measuring the moisture of the rice grains, the samples were examined with regard to fungal contamination. In this study, 4 common rice mycotoxins, including aflatoxin, ochratoxin, zearalenone and deoxynivalenol were examined with the HPLC method. The resulted data were statistically analyzed with the SPSS software.

Results:
The mean moisture of the rice grains was 11.59±0.53. Laboratory analysis showed that of the examined 123 samples, 34 (27.6%) had deep contamination with the fungal invasion of the tissues of rice. The highest contamination was related, respectively, to aspergillus niger (7 cases, 5.7%), aspergillus flavus (5 cases, 4.1%), and penicillium and candida (each 4 cases, 3.3%). Neither of the samples showed mycotoxin contamination over the allowable limit.

Conclusion:
The findings showed that despite the fungal contamination of the samples, mycotoxin contamination was null or close to null in all cases. This result was compatible with the reports of the country’s Standard Organization about the mycotoxin contamination of the imported rice.

Keywords: Mycotoxins, Ochratoxins, Zearalenone, deoxynivalenol, Fungus Diseases, Rice

Introduction
Agricultural crops, such as oilseeds, nuts, dried fruit, and most of the grains, are prone to fungal contamination. The three fungi contaminating rice are as follows: aspergillus, fusarium, and penicillium. These fungi invade crops and cause changes in color, reduction in marketability, fragility, vulnerability, and contamination with mycotoxins (1 & 2).
Despite the inherent resistance of rice against fungal contaminations, it is exposed to fungal contamination during planting, growing, harvesting, and storing. An important underlying cause of contamination of rice is its moisture content. Results of various studies show that aflatoxin and aspergillus contaminations are directly correlated with moisture content of the stored rice (3).

A persistent downpour in wet areas exposes rice to fungal and bacterial invasion and contamination, especially in warm areas. Although farmers try to dry the crop in the sun in warm seasons, moisture of grains is not reduced to a reasonable amount. Crops with high moisture cannot be warehoused appropriately and lead to fungal contamination during harvesting and warehousing. Therefore, climatic conditions and also the quality of warehousing are two important factors making rice contaminated with mycotoxins (3 & 4). Under optimum growing conditions, the contamination leads to mycotoxin production. Based on World Health Organization's estimations, one fourth of the crops produced every year are contaminated with mycotoxins, and the incurred losses and damages to the agricultural crops cost 100 million dollars.

Mycotoxins affect animal products unfavorably besides their adverse effect on agricultural products. Economic losses, including economic damages to animal husbandry, animal and poultry mortality, reduced laid eggs, reduced fertility, prevalence of animal diseases in animal farms and poultry farms, weakened immune system of animals, reduced rate of growth and reproduction, increased food conversion ratio, and increased costs of planning for reducing risks. Moreover, milk, eggs, and meat of these animals may contain the residual mycotoxin (5).

Mycotoxins are resistant compounds circulating in nature for a long time and enter human body through the food chain. Although the amount of mycotoxins entered human body is very little, their adverse effects appear after a long time and cause various disorders, including cancers and hepatic, gastrointestinal, hematologic, and renal disorders due to the cumulative effect of mycotoxins (6 & 7). Among various mycotoxins contaminating rice, aflatoxin B1 and ochratoxin A have maximum toxic effect on mammals and cause disorders such as hepatitis, hemorrhage, edema, immune system suppression, liver carcinoma and, renal toxicity, with their hepatotoxic, teratogenic, and mutagenic properties. International Agency for Research on Cancer classified aflatoxin B1 under group 1 and fumonisins B1 and B2 under group 2 of human carcinogens (5 & 8). Global spread of mycotoxins in rice and grains and their toxicity for humans and animals have necessitated measurement of these toxins in grains (9). According to the documents of Iran’s National Standard Organization, upper limit for aflatoxin B1, all types of aflatoxins, ochratoxin, deoxynivalenol, and zearalenone is 5 ng/g, 30 ng/g, 5 ng/g, 1000 ng/g, and 200 ng/g respectively. Contamination of grains, especially rice, with mycotoxins, their broad adverse effects on different body organs in humans and animals, and the resulting economic losses have made the contamination an important problem in food hygiene and general health in the society. Numerous studies conducted in Iran on contamination food with various types of mycotoxin confirm the importance of the problem (10, 11, & 12).

Regarding the foregoing, the special position of rice in the food basket of Iranian households, and high consumption of rice and its derivatives in Iran, this study was conducted to examine imported rice consumed in Zabol, Iran in terms of mycotoxin contamination and its producing fungi.

Materials and Methods
In this study, 123 samples of rice were collected from rice sale centers in Zabol.
using convenience sampling method. Rice samples of 150-200 g were removed from the middle of rice supplies, using stratified sampling method, and poured into the sterile paper bags. After collecting relevant data, samples were kept in plastic bags.

To measure moisture content of rice, once the samples were ground, 5 g of each sample was heated in an oven (Memert model, made in Germany) at 105°C for 3 hours. Then, samples were cooled in a desiccator and the difference in weight of the crucible before and after heating was measured.

To examine fungal contamination of samples, 15 rice grains with signs of tissue damages as discoloration and fragility were selected from each sample and washed out several times with sterile distilled water to remove residual sodium hypochlorite after being disinfected superficially with 1% sodium hypochlorite solution. The grains were first chopped in sterile conditions under a laboratory hood with a sterile scalpel and then cultivated in plates containing the culture medium (YGC) (13). To better isolate rice grains cultivated in the culture medium, five rice grains were cultivated in each plate, and totally, fifteen grains of each sample were cultivated in three plates. Riddle’s slide culture method was used to determine the exact identity of isolated fungi. The plates were incubated at 25°C up to 30 days. Having checked the plates during this period, fungal colonies were detected and counted, and the percentage of contamination, the number of grown fungal colonies, and also the type of the contaminating fungi were determined. The contaminating fungi were detected on the basis of morphologic characteristics, structure of sporulation, and biochemical tests (14 & 15). Frequency and central indicators of the data related to the rate of contamination and genera and species of contaminating fungi were determined. Pearson’s chi-squared test was used to test mean differences in contamination of different samples and compare them with one another.

Rice samples of 150-200 g collected from rice supplies of sale centers in Zabol were first examined for fungal contamination and then classified into four main categories by type of the crop and integrated together in a way that 4-5 kg of rice were collected for each category. The integrated rice categories were ground and were examined in terms of contamination using reversed-phase high-performance liquid chromatography (HPLC). The HPLC device used in this study was HPLC Agilent Technologies 1200 Series made in Germany.

**Measurement of aflatoxins**

Aflatoxins were measured using HPLC that was based on Iranian National Standard no. 6872 and consisted of three steps of separation, detection, and determination. Separation was done using reversed-phase column; detection was done using Kobra Cell method; and determination was performed using fluorescence detector, comparing the area under the standard curve with that of the unknown sample, and measuring the dilution coefficient. In this regard, once the samples were ground, 50 g of the ground rice was mixed with 5 g of NaCl and 200 ml of 80% methanol within three minutes. Once the obtained extract was passed through Whatman filter paper, grade 1, 20 ml of the filtered extract was mixed with 130 ml of water and passed through fiberglass filter paper (mesh 1.6 µm). Of the extract, 75 ml was passed through the immunoaffinity column at the speed of 2-3 ml/min equaling to one drop per second. Then, 1500 µl of methanol was passed through the column in order to collect the residual extract to the last drop in a clean vial. The content of the vial was diluted with 1500 µl of water and well mixed by vortex. Working standard solution with a volume of 200 µl was injected to the HPLC device, and the calibration curve

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was drawn accounting of the standard changes in mass of the toxin (ng) on X-axis and changes in the area under the curve on the Y-axis. Then, the final extract of the sample with volume of 100 µl was injected to the HPLC device, and the rate of contamination was calculated using the calibration curve, comparing the area under the curve or the height of standard curves with that of the unknown samples, and applying dilution coefficient. Fluorescence detector with an excitation wavelength of 365 nm and emission of 435 nm was used to detect aflatoxin.

**Measurement of ochratoxin**

Ochratoxins was measured according to the Iranian National Standard no. 9238. Separation, detection, and determination through HPLC were respectively done using reversed-phase column, Kobra Cell method, and fluorescence detector with an excitation wavelength of 333 nm and emission of 477 nm, and comparing the area under the standard curve with that of the unknown sample taking into account the dilution coefficient. To do so, once the samples were ground, 25 g of the ground rice was mixed with 1 g of NaCl and 100 ml of solvent extraction of ochratoxin within three minutes. Once the obtained extract was passed through Whatman filter paper, grade 1, 10 ml of the filtered extract was diluted with 50 ml of PBS solution and passed through fiberglass filter paper (mesh 1.6 µm). Of the extract, 55 ml was passed through the column specific for ochratoxins with the speed of 2-3 ml/min equaling to one drop per second. The toxin connected with the antibody inside the column was washed out by passing 1500 µl of methanol through the column, collected into a clean vial, and diluted with 1500 µl of water. To measure the toxin, working standard solution with a volume of 100 µl was firstly injected to the HPLC device, and the calibration curve was drawn. Then, the final extract of the sample with volume of 100 µl was injected to the HPLC device, and the rate of contamination was calculated using the calibration curve, comparing the area under the curve or the height of standard curves with that of the unknown samples, and applying dilution coefficient.

**Measurement of zearalenone**

Zearalenone was measured based on the Iranian National Standard no. 9239. Once the rice samples were ground, 25 g of the ground rice was mixed with 1 g of NaCl and 100 ml of solvent extraction of zearalenone within three minutes. The obtained extract was passed through Whatman filter paper, grade 1, and 10 ml of the filtered extract was diluted with 65 ml of water and passed through fiberglass filter paper (mesh 1.6 µm). Of the filtered extract, 65 ml was diluted and passed through the immunonaaffinity column with antibodies specific for zearalenone at the speed of one drop per second. The toxin connected with the antibody inside the column was washed out by passing 2000 µl of methanol through the column and collected into a clean vial to the last drop. The content of the vial was diluted with 2000 µl of water and mixed by vortex. Separation, detection, and determination were respectively done using reversed-phase chromatography, ultraviolet detector at a wavelength of 275 nm or fluorescence detector with an excitation wavelength of 275 nm and emission of 450 nm, and comparing the area under zearalenone standard curves with that of the unknown sample and accounting of the dilution coefficient. To measure the toxin, zearalenone working standard solution with a volume of 200 µl was injected to the HPLC device, and the calibration curve was drawn. Then, the final extract of the sample with volume of 150 µl was injected to the HPLC device, and the rate of contamination was calculated using the calibration curve, comparing the area under the curve or the height of standard curves with that of the unknown samples, and applying dilution coefficient.
**Measurement of deoxynivalenol**

Deoxynivalenol was measured according to the Iranian National Standard no. 10215. In this regard, once the rice samples were ground, 25 g of the ground rice was mixed with 1 g of NaCl and 100 ml of DON solvent extraction within three minutes. The obtained extract was passed through Whatman filter paper, grade 1, and 5 ml of the filtered extract was passed through DONSPE columns and collected into a vial. To wash out the column, 2.5 ml of 84% acetonitrile solution was passed through the column and collected into the same vial (the vial content reached 7.5 ml). The vial was placed in a bain-marie at 40-50°C and dried completely in the vicinity of the air pressure (vacuum pump). Then, 1 ml of deoxynivalenol mobile phase solvent was added to the vial and mixed by vortex for 1 min and a sonicator for 1 min. The vial content was mixed again by vortex for 1 min.

To measure the toxin, deoxynivalenol working standard solution with a volume of 50 µl was injected to the HPLC device, and the calibration curve was drawn. Then, the final extract of the sample with volume of 200 µl was injected to the HPLC device, and deoxynivalenol was detected using ultraviolet detector at wavelength of 218 nm. The resulted peaks were compared to the standard peak, and the rate of contamination was calculated using the calibration curve, comparing the area under the curve or the height of standard curves with that of the unknown samples, and applying dilution coefficient.

**Results**

In this study, 123 samples of four types of rice mainly imported to Zabol were collected and examined. The rice imported to Zabol was mainly imported from India and Pakistan. Based on the information obtained from retailers and wholesalers, the rice was imported by land and by sea to Sistan-Baluchestan Province, and the brand recorded on rice bags was related to packaging companies and determined the quality of rice to some extent.

The allowed moisture content for rice is maximally 14% based on the available standards. In this study, mean moisture content of the studied samples was 11.59%±0.53%.

Table 1 provides contamination rate of samples for different types of rice (Table 1).

<table>
<thead>
<tr>
<th>Item</th>
<th>Type of sample</th>
<th>Contaminated rice</th>
<th>Non-contaminated rice</th>
<th>Statistical test results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Number</td>
<td>Percentage</td>
<td>Number</td>
</tr>
<tr>
<td>1</td>
<td>Pakistani white rice</td>
<td>11</td>
<td>34.4%</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>Pakistani yellow rice</td>
<td>8</td>
<td>26.7%</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>Indian yellow rice</td>
<td>7</td>
<td>21.9%</td>
<td>25</td>
</tr>
<tr>
<td>4</td>
<td>Thai white rice</td>
<td>8</td>
<td>27.6%</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>Total</td>
<td>34</td>
<td>27.6%</td>
<td>89</td>
</tr>
</tbody>
</table>

Results of the statistical test showed no significant difference between various types of rice in terms of fungal contamination (P=0.736 & df=3).

However, maximum and minimum percentage of contamination was related to Pakistani white rice (34.4%) and Indian yellow rice (21.9%), respectively.

Laboratory examination of the samples showed that 34 samples (27.6%) out of the 123 samples were contaminated, as the highest contamination rates were respectively related to aspergillus niger (7 samples, 5.7%), aspergillus flavus (5 samples, 4.1 %), and penicillium and candida (each with 4 samples, 3.3%). Figure 1 shows frequency distribution of genera and species of fungi contaminating rice imported to Zabol.
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Measurement of mycotoxins

Figure 1 shows the chromatogram obtained for the four types of aflatoxin. The measurement was done using fluorescence detector at 435 nm and 365 nm wavelengths.

According to the results, the amount of aflatoxin B1 and the total aflatoxin in the Pakistani sample was 1.02 ng/g and 1.14 ng/g, respectively. Moreover, the total amount of aflatoxin in Pakistani light yellow rice, Hindi light yellow rice, and Thai white rice was 0.35 ng/g, 0.75 ng/g, and 0.11 g, respectively. The results indicated that the amount aflatoxin in all samples was within permissible limit for rice although the considerable amount of aflatoxin B1 in Pakistani white rice sample compared to other aflatoxins should receive special attention.

Figure 2 shows the calibration curve and the chromatogram of ochratoxin.

Figure 1: Frequency distribution of genera and species of fungi contaminating rice imported to Zabol fungal contamination

(left to right): Aspergillus flavus, Aspergillus fumigates, Aspergillus parasiticus, Aspergillus niger, Penicillium, Fusarium, Bipolaris, Curvularia, Helminthosporium, Rhizopus, Geotrichum)

Figure 1: The chromatogram obtained through HPLC for aflatoxins B1, B2, G1, and G2 in Al rice sample
The results showed that the amount of ochratoxin was not detectable by the device even at LOQ=0.790 ppb, and ochratoxin did not exist in the rice samples.

To measure zearalenone, firstly, zearalenone working standard solution with certain concentrations was injected to HPLC device to draw the calibration curve, and then, unknown samples were measured using fluorescence detector at 450 nm and 275 nm wavelengths. Figure 3 shows the calibration curve and the chromatogram of zearalenone.

The results showed no zearalenone in the four types of rice, as it was not detectable by the device even at LOQ=18.400 ppb.

To measure deoxynivalenol, firstly, deoxynivalenol working standard solution with certain concentrations was injected to HPLC device, and the calibration curve was drawn. Then, unknown samples were measured using ultraviolet detector at 218 nm wavelength. Figure 4 shows the calibration curve obtained from injection of standard solutions and regression of the figure and chromatogram of deoxynivalenol.

The results showed no deoxynivalenol in the four types of rice. Deoxynivalenol was not detectable by the device even at LOQ=293.600 ppb.
Discussions
According to the existing standards, the permissible moisture content for rice is maximally 14%. In this study, mean moisture content of the studied samples was within the standard range (11.59%±0.53%), and could be as a key factor effective in reduction of fungal contamination and mycotoxin production in the crop.

The results showed that the prevalence of aspergillus flavus was higher than that of aspergillus parasiticus. The difference might be due to the low moisture of the samples (11.59%±0.53%) in this study. In other words, ideal conditions were not prepared for growth of aspergillus parasiticus.

Maximum contamination of rice with aspergilli was reported at the time of warehousing, especially when rice weevil increased (16). Reddy et al. and Raghavender et al. showed the invasion of insects and increased aspergillus flavus contamination (17 & 18). The present study indicated contamination of rice with weevil (1.6%) in two. This kind of contamination might be due to the inappropriate long-term warehousing of rice.

Although aspergilli were removed from rice grains, aflatoxin contamination was very low (19). However, discolored contaminated rice faced with marketability problems, and consequently, its cost and quality were reduced. A study using electron microscope indicated the invasion of aspergillus fungal agents to different parts of rice grains (20).

Each fungal agent can invade rice under certain ecosystem conditions. Aspergilli in the rice warehoused at high humidity cause maximum contamination. However, a major part of the contamination was the superficial colonization that was washed out with disinfectants, and consequently, the fungal contamination in rice was measurable with a frequency very lower than that before (21 & 22). In this study, the comparison of superficial contamination of samples (98 cases, 79.6%) with contamination of deep tissues of rice grains (34 cases, 27.6%) could be a proof for superficial colonization of fungal spores. Risks of colonization would be eliminated when the rice is cooked before consumption, but if the inappropriate conditions of warehousing pave the way for invasion of fungi to internal tissues of rice, the probability of mycotoxin production and its adverse effects would increase. Regarding the few studies in this regard, no comprehensive study has been conducted on different parts of rice in terms of fungal flora and fungal contamination in various ecosystems. The results revealed that the rate of mycotoxin contamination in all samples was zero or almost zero. This result conformed to the reports of Iran’s National Standard Organization about lack of mycotoxin contamination of imported rice.
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