Quantification of Aflatoxins in Different Dates (Phoenix Dactylifera L.) Varieties from Humid Subtropical Regions

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Abstract
Food items are prone to fungal attacks due to the warm and humid climatic conditions of Pakistan. The aflatoxins (AFs) are the most common mycotoxins produced by fungal species highly responsible for liver toxicity and hepatocellular carcinoma. The present study was planned to assess the contamination levels of aflatoxins (AFB1, AFB2, AFG1, and AFG2) in local and imported varieties (Irani and Saudi) collected from Pakistan. About 251 dates samples were procured from the markets and analyzed by reverse-phase high-performance liquid chromatography coupled with a fluorescence detector (HPLC-FLD) in isocratic mode. The AFs were detected in 89% of date samples ranging from 32.9 to 1465.4 μg kg⁻¹ all of which exceeded the permissible limit of AFs set by FDA (USA) (20 μg kg⁻¹) and EU (4 μg kg⁻¹) for human consumption. The two main local varieties Kupro (801.5 μg kg⁻¹), and Mazafati (1275.8 μg kg⁻¹) had maximum concentrations of total aflatoxins. Out of imported varieties, Mabroom (Saudi) (272.94 μg kg⁻¹) and Rubai (Irani) (1465.42 μg kg⁻¹) have the highest concentrations of total aflatoxins. The maximum levels of AFB1 were seen in Mazafati (Pakistani) (521.5 μg kg⁻¹), Mabroom Saudi (127.6 μg kg⁻¹) and Rubai Irani (662.8 μg kg⁻¹). It could be seen that overall local varieties have higher contamination of AB1 and total AFs with the exception of Rubai (Irani) and Mabroom (Saudi) due to the poor storage conditions, improper pre- and post-harvesting handling, and humid conditions of Pakistan emphasizing the need for proper management and regulation.

Keywords: Aflatoxin, dates varieties, districts, Pakistan, HPLC-FLD, EU limits


1. Introduction

Date Palm (Phoenix dactylifera L.) belongs to a family Arecaceae and bears a fruit known as dates [1]. Dates are a rich source of potassium, sodium, magnesium, fiber, carbohydrate, protein, and a very less percentage of fat [2]. The daily use of dates may protect humans from cellular damage, high blood pressure, and cardiovascular diseases due to the presence of high antioxidants [3]. This plant occurs profoundly in the areas of North Africa, Southwest Asia, and South Asia [4]. The environmental conditions of Pakistan are suitable for the cultivation of Date Palm with worth production of 0.60 million metric tons [5]. Date fruit near the maturity stage or during processing, transport, and storage are vulnerable to fungal attack due to high moisture content [6,7]. Mycotoxins are organic compounds, having molecular weight >500 Da produced by well-known species of fungi like Aspergillus, Penicillium, and Fusarium. More than 600 types of mycotoxins have been identified but aflatoxins are the most poisonous form and unavoidable contaminants present in a variety of foods thus are most frequently studied [7,8,9,10]. Aflatoxins (AFB1, AFB2, AFG1, AFG2) are mostly produced by Aspergillus flavus, A. parasiticus and seldom by A. nomius [7,11] contaminating multi-variety of foods i.e., rice grains, maize, milk, peanut, chilies [12,13,14,15]. Among, aflatoxins, aflatoxin B1 (AFB1) is recognized as the utmost lethal, severe, and carcinogenic [7] and
International Agency for Research on Cancer has ranked it as human carcinogen I [16].

The frequency and degree of aflatoxins contamination are influenced by different elements such as humidity, temperature, physical damage, concurrent mycobiota, water activity, and other storage conditions. High temperature coupled with periods of drought exaggerates the production and transfer of aflatoxins from field to fork. Consequently, the mainstream of the world’s population (approximately 4.7 billion people) living in the hot zone of the world are frequently exposed to aflatoxins. The environmental conditions (temperature, moisture, and humidity) in tropical countries like Pakistan may be conducive for the invasion of fungi and it can produce aflatoxin in food [17]. A very high concentration of aflatoxins was found in nuts and dry fruits procured from Pakistan [7,18]. A similar kind of study was initiated in Turkey to explore the aflatoxins levels in hazelnuts, walnuts, peanuts, almonds, roasted chickpeas and listed a very high prevalence of total aflatoxins (1-133 µg/kg) with 133 µg/kg in a single hazelnut [19]. The concentration of aflatoxins was evaluated in nuts consumed in Tehran, Iran during 2013 using ELISA techniques and 96.5% samples were found contaminated with aflatoxins [20].

Dates have been grown and exported to other parts of the world and these are very susceptible to fungi and subsequent production of aflatoxins. Moreover, there has not been any regulation limit set for aflatoxins in Pakistan. Hence, it is important to explore the contamination levels for aflatoxins and aid law enforcement agencies to execute strict guidelines for aflatoxins. In view of the above-mentioned facts, the current research was planned to explore the contamination levels of aflatoxins in different imported and local dates varieties sold in markets of Pakistan and compared the concentration of aflatoxins with established maximum limits of EU and FDA. The generated data will be of great importance for the food authority for the safety of the end-users.

2. Materials & Methods

2.1. Experimental Location and Sampling

A total of 251 date samples of 11 local and 10 imported dates varieties were collected randomly from different sites of five main divisions of Punjab, Pakistan during September 2019-February 2020 (Figure 1).

The selected sites were more than 500 meters away from each other. The details of date varieties (local and imported) are given in Table 1. About 1 kg samples were purchased from different retail markets, superstores, local shops, and wholesale markets, packed in a zipper bag with code of identification and put into thermophore icebox (4±2°C) and brought to Food Toxicology Laboratory at Nuclear Institute for Agriculture and Biology, Pakistan. The stones were manually separated and flesh was homogenized using high speed blender (Braun Multipurpose, Germany) and individually stored at -4°C until further investigation.

![Figure 1. Sampling of dates from five divisions of Punjab, Pakistan](image-url)
Table 1. Incidence and mean levels of AFB1 and total aflatoxins in local and imported date varieties from Pakistan

<table>
<thead>
<tr>
<th>Dates varieties</th>
<th>Samples (n)</th>
<th>Positive (%)</th>
<th>AFB1 (µg/kg)</th>
<th>Total aflatoxins (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local</td>
<td>138</td>
<td>104</td>
<td>206.33</td>
<td></td>
</tr>
<tr>
<td>Aseel</td>
<td>15</td>
<td>13</td>
<td>96.47</td>
<td>206.33</td>
</tr>
<tr>
<td>Barni</td>
<td>9</td>
<td>5</td>
<td>239.68</td>
<td>585.36</td>
</tr>
<tr>
<td>Jamshoro</td>
<td>15</td>
<td>10</td>
<td>65.70</td>
<td>142.02</td>
</tr>
<tr>
<td>Kalbi</td>
<td>9</td>
<td>4</td>
<td>23.45</td>
<td>52.86</td>
</tr>
<tr>
<td>Karbalyne</td>
<td>12</td>
<td>9</td>
<td>86.12</td>
<td>179.45</td>
</tr>
<tr>
<td>Kupro</td>
<td>15</td>
<td>15</td>
<td>318.48</td>
<td>801.55</td>
</tr>
<tr>
<td>Mazafati</td>
<td>15</td>
<td>15</td>
<td>521.52</td>
<td>1275.85</td>
</tr>
<tr>
<td>Panjgeo</td>
<td>6</td>
<td>3</td>
<td>37.48</td>
<td>60.85</td>
</tr>
<tr>
<td>Paymaro</td>
<td>12</td>
<td>11</td>
<td>112.57</td>
<td>237.91</td>
</tr>
<tr>
<td>Rabai</td>
<td>15</td>
<td>12</td>
<td>75.53</td>
<td>155.71</td>
</tr>
<tr>
<td>Shangoo</td>
<td>15</td>
<td>7</td>
<td>90.52</td>
<td>181.65</td>
</tr>
<tr>
<td>Import Saudi</td>
<td>54</td>
<td>42</td>
<td>221.48</td>
<td></td>
</tr>
<tr>
<td>Ajwa</td>
<td>15</td>
<td>12</td>
<td>107.71</td>
<td></td>
</tr>
<tr>
<td>Kalma</td>
<td>12</td>
<td>10</td>
<td>120.89</td>
<td>257.32</td>
</tr>
<tr>
<td>Mahroom</td>
<td>15</td>
<td>13</td>
<td>127.56</td>
<td>272.94</td>
</tr>
<tr>
<td>Sugai</td>
<td>6</td>
<td>3</td>
<td>40.84</td>
<td>94.29</td>
</tr>
<tr>
<td>Sukhari</td>
<td>6</td>
<td>4</td>
<td>61.60</td>
<td>131.44</td>
</tr>
<tr>
<td>Import Irani</td>
<td>59</td>
<td>51</td>
<td>236.78</td>
<td></td>
</tr>
<tr>
<td>Payaram</td>
<td>15</td>
<td>15</td>
<td>109.46</td>
<td></td>
</tr>
<tr>
<td>Ringro</td>
<td>12</td>
<td>10</td>
<td>324.70</td>
<td>780.65</td>
</tr>
<tr>
<td>Rubai</td>
<td>5</td>
<td>3</td>
<td>662.78</td>
<td>1465.42</td>
</tr>
<tr>
<td>Sharifa</td>
<td>15</td>
<td>13</td>
<td>125.83</td>
<td>285.53</td>
</tr>
<tr>
<td>Zahidi</td>
<td>12</td>
<td>10</td>
<td>107.84</td>
<td>272.79</td>
</tr>
</tbody>
</table>
| **Total**       | **251**     | **197 (89%)**| **2.2. Chemicals and Reagents**

The standards for aflatoxins (AFB1, AFB2, AFG1, and AFG2) were procured from Sigma-Aldrich, Steinheim, Germany in solid form. The clean-up column MycoSep 226 was obtained from Romer Labs Inc., Sandy Drive, NY, USA. Acetonitrile (CAS # 75-05-8), methanol (CAS # 67-56-1), and trifluoroacetic acid (CAS # 76-05-1) of analytical grade obtained from Merck, Darmstadt, Germany. Double distilled water was prepared using Water Stills, Bibby W40, England.

2.3. Extraction and Determination of Aflatoxins

The extraction of aflatoxins from date samples was performed following the previously validated method [18] with some modifications. For this purpose, a 25 g ground sample of dates was taken in 250 ml glass Erlenmeyer flask, 100 ml (acetonitrile:H2O; 84:16 v/v) with 2% sodium chloride was added. The flasks were shaken at medium speed (60 rpm) using a horizontal shaker (SHO-2D, Nisd Laboratory Instrument, China). The content (9 mL) was filtered and acidified with acetic acid (70 µL) and the cleaned contents (2 mL) were collected after passing through MycoSep 226 clean up column, Romer Laboratories (Union, MO, USA). The content was evaporated (N2 gas) and derivatized with trifluoroacetic acid. The aflatoxins residues were re-dissolved in 1.95 mL (acetonitrile: H2O; 9:1, v/v) and stored (4°C) for chromatographic analysis.

The HPLC analysis was performed using a Shimadzu LC-10 series (Japan) coupled with a binary delivery pump (LC-10AS), column oven (CTO-10A), system controller (SCL-10A), a manual sampler (Rheodyne, USA), and a fluorescence detector (RF-530) set at 365 nm (Excitation) and 440 nm (Emission). Isocratic elution was done at a flow rate of 1.5 ml/min using the mobile phase composed of acetonitrile/methanol/water (22.5:22.5:55 v/v/v). The temperature was fixed at 30°C and the injection volume was 20 µL. Discovery C18 column (4.6 × 250 mm, 5 µm particle size, Supelco, Bellefonte, PA, USA) was used as a stationary phase without the guard column. The total run time of the analysis was 7 mins and the results were documented by means of the CLASS LC 10 software through communication bus module (CBM-101). For calibration curve, standard solutions of 0.1, 0.5, 1, 2.5, 5, and 10 µg/mL were made from the stock solution (1000 mg/L) of individual aflatoxins (AFB1, AFB2, AFG1, and AFG2) and average peak areas were calculated. A blank (check) sample was run before proceeding samples to get a smooth baseline without any interference (Figure 2, Figure 3).
2.4. Method Validation

The established method was validated according to the guidelines provided by IUPAC and Eurachem [21,22]. The subsequent certified factors (linearity, recovery %, limit of detection (LOD), limit of quantification (LOQ), sensitivity, ruggedness, precision, and accuracy) were studied. The recovery % was determined by spiking dates samples at different concentrations, extracted, purified, and analyzed after flushing the HPLC system with methanol (HPLC grade) for one hour. The obtained concentration was calculated and recorded along with other performance parameters. The precision and accuracy of the method were assessed as repeatability and reproducibility at three different concentration levels within the same day by means of a triplicate analysis of the spiked samples (Table 2).

Analytical procedure for aflatoxins was performed for linearity (Table 3), 0.5-5 μg/kg aflatoxin B1 and G1, and 0.1-10 μg/kg aflatoxin B2 and G2. The limit of detection (LOD) was expressed for HPLC sensitivity as estimated as signal/noise ratio (S/N, 3:1) [23]. The spiking standard solutions were used to evaluate method accuracy through recovery experiments.

Afterward, the precision and accuracy was calculated using the standard deviation (SD) and relative standard deviation (RSD) (Table 3). The ruggedness of the applied procedure has been categorized as the resistance to the changes in the results of any analytical method when any kind of deviation was done in the experimental conditions [21]. The ruggedness can be significantly affected due to various factors like the quality of the stationary phase, LOD, LOQ, or changes in the retention time. Furthermore, the content of aflatoxins in the samples was examined using analytical columns of varying length, particle size of the stationary phase, and internal diameter.

2.5. Statistical Analysis

Results of the current study are stated in the form of mean ± standard deviation or as a percentage. Statistical analysis was performed by using Microsoft Excel version 365. The acquired data underwent to one-way analysis of variance (ANOVA) and the values were regarded significantly different when the value of p < 0.05.

3. Results and Discussion

3.1. Method Performance

Analytical method based on HPLC was assessed for quality assurance using core factors such as recovery, linearity, sensitivity (limit of detection LOD and limit of quantification LOQ), and precision (repeatability and reproducibility). Linearity was performed by injecting aflatoxins standard into HPLC-fluorescence ranging 0.05 to 5 μg/L for AFB1 and AFG1, 0.1 to 10 μg/L for AFB2 and AFG2 respectively. The correlation coefficients acquired for aflatoxins (AFB1, AFB2, AFG1 & AFG2) were 0.9930, 0.9999, 0.9999, and 0.9921 respectively (Table 2). Recovery studies were achieved from spiked samples at different aflatoxins concentrations (1, 5, 10, 20, and 50 μg/kg). The samples were processed after 1 h and analyzed by HPLC isocratically. The mean recoveries for dates (local and imported) were 95 ± 1.18, 92 ± 1.15, 95 ± 1.01, and 92 ± 1.74, respectively.

Intra-day (n=5) and inter-day (5 different days) studies were performed to assess repeatability and reproducibility. The results revealed values of repeatability (relative standard deviation, RSDr) and reproducibility (RSDR) ranged 5.9% and 6.18% respectively that exhibiting a fair precision (Table 4). The detection limit (LOD) and the limit of quantification (LOQ) values were intended according to s:n=3 and s:n=10, respectively. The LODs and the LOQs of AFB1 and AFG1; AFB2 and AFG2 were 0.045 μg/kg; 0.12 μg/kg and 0.36 μg/kg, respectively.

Convincingly, the studied method satisfied the performance standards of Commission Regulation (EC) No. 401/2006 for the precise confirmation of aflatoxins concentrations in food commodities. According to EC 401/2006, the combined samples of dates shall be at least 1 kg [24].

### Table 2. Parameters of linear regression measured for aflatoxins (AFB1, AFB2, AFG1, and AFG2) in HPLC

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>Concentration</th>
<th>Slope (a)</th>
<th>Intercept (b)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB1</td>
<td>0 - 5 μg mL⁻¹</td>
<td>77558</td>
<td>15720</td>
<td>0.993</td>
</tr>
<tr>
<td>AFB2</td>
<td>0 - 10 μg mL⁻¹</td>
<td>121000</td>
<td>1471</td>
<td>0.9999</td>
</tr>
<tr>
<td>AFG1</td>
<td>0 - 5 μg mL⁻¹</td>
<td>41916</td>
<td>1307</td>
<td>0.9999</td>
</tr>
<tr>
<td>AFG2</td>
<td>0 - 10 μg mL⁻¹</td>
<td>111847</td>
<td>26844</td>
<td>0.9921</td>
</tr>
</tbody>
</table>

*y = ax + b; y = peak area, x = ng injected, R² = regression coefficient.

### Table 3. Quality parameters of HPLC for the analysis of aflatoxins

<table>
<thead>
<tr>
<th>Aflatoxin</th>
<th>Retention time (min)</th>
<th>Linearity (μg mL⁻¹)</th>
<th>LOD (ng mL⁻¹)</th>
<th>LOQ (ng mL⁻¹)</th>
<th>Precision (%RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFB1</td>
<td>2.89 ± 0.04</td>
<td>0.05 - 5</td>
<td>0.045</td>
<td>0.135</td>
<td>5</td>
</tr>
<tr>
<td>AFB2</td>
<td>3.96 ± 0.04</td>
<td>0.1 - 10</td>
<td>0.12</td>
<td>0.36</td>
<td>6</td>
</tr>
<tr>
<td>AFG1</td>
<td>2.66 ± 0.04</td>
<td>0.05 - 5</td>
<td>0.045</td>
<td>0.135</td>
<td>6</td>
</tr>
<tr>
<td>AFG2</td>
<td>3.40 ± 0.03</td>
<td>0.1 - 10</td>
<td>0.12</td>
<td>0.36</td>
<td>5</td>
</tr>
</tbody>
</table>

RSD: relative standard deviation; LOD: limit of detection; LOQ: limit of quantification.
3.2. Occurrence of Aflatoxins in Dates

Prevalence and contamination level of aflatoxins in different date varieties collected from different districts of Punjab province, Pakistan are presented in Table 1. The results showed that out of 221 samples (local and imported), 197 (89%) samples were contaminated with aflatoxins. The 75% local date samples and 37% imported date varieties were contaminated. In two local varieties (Kupro, and Mazafati) of dates, the prevalence of AFB1 was 100% with total aflatoxins 801 to 1275 µg/kg while other varieties contained total aflatoxins ranging from 53 to 238 µg/kg. Conversely, the differences between date varieties were significant (p<0.05). Moreover, there were also significant differences in AFB1 levels subject to date variety (p<0.1). Among local dates varieties, Mazafati contained the highest concentration of AFB1 (521.52 µg/kg) and total aflatoxins (1275.85 µg/kg) while dates collected from other cities exhibited occurrence 75.3% with lower levels of AFB1 between 23.45 to 110 to 180 µg/kg, the concentration matched with the maximum limit set by EU with the maximum occurrence of AFB1 having mean levels of 4.8 to 6.2 µg/kg. However, in our study, 75% of local date varieties were positively contaminated with AFB1 having mean levels of 4.8 to 6.2 µg/kg. Moreover, flavor, color, and chemical changes that occur during the ripening stages of fruit makes the dates more susceptible to the attack of Aspergillus flavus [27]. Different studies have reported incidence of aflatoxins in dates and its products (Table 5).

Reference [26] observed noticeable levels of AFB1 and AFB2 in three out of the 25 dates varieties of U.A.E and stored products that were kept for 14 days at 30°C and 98% relative humidity. No aflatoxins were detected in the Tamer stage however, in our study the samples were positive for AFB1 at Tamer stage. Reference [28] have stated the incidence of AFs in forty date samples of varying categories including paste, dried, raw, peanut stuffed, almond and fumigated using Thin layer chromatography and observed two out of the five samples of pitted date fruits stuffed with peanut to be contaminated by AFB1 with mean concentrations from 4.8 to 6.2 µg/kg. However, in our study, 75% of local date varieties were contaminated with aflatoxins having the range from 53 to 1275 µg/kg. Reference [29] have explored twenty samples of date for the analysis of AFB1 and two of them were positively contaminated with AFB1 having mean levels of 110 to 180 µg/kg, the concentration matched with the current findings of AFB1 levels in dates.

Our study was also in accordance with the studies of [7,18,30,31,32] who reported contaminated samples to be above the recommended limits of EU. Furthermore, most of the studies were conducted in the Middle East countries like U.A.E, Saudi Arabia, and Egypt and also from Pakistan, where the climatic conditions like temperature of 30°C and relative humidity higher than 90% are the optimum conditions for the fungal outbreak in fresh dates [26]. Moreover, flavor, color, and chemical changes that occur during the ripening stages of fruit makes the dates more susceptible to the attack of Aspergillus flavus [27]. Different studies have reported incidence of aflatoxins in dates and its products (Table 5).

Reference [26] observed noticeable levels of AFB1 and AFB2 in three out of the 25 dates varieties of U.A.E and stored products that were kept for 14 days at 30°C and 98% relative humidity. No aflatoxins were detected in the Tamer stage however, in our study the samples were positive for AFB1 at Tamer stage. Reference [28] have stated the incidence of AFs in forty date samples of varying categories including paste, dried, raw, peanut stuffed, almond and fumigated using Thin layer chromatography and observed two out of the five samples of pitted date fruits stuffed with peanut to be contaminated by AFB1 with mean concentrations from 4.8 to 6.2 µg/kg. However, in our study, 75% of local date varieties were contaminated with aflatoxins having the range from 53 to 1275 µg/kg. Reference [29] have explored twenty samples of date for the analysis of AFB1 and two of them were positively contaminated with AFB1 having mean levels of 110 to 180 µg/kg, the concentration matched with the current findings of AFB1 levels in dates.

The concentrations were mean ± standard deviation; RSD: relative standard deviation.
4. Conclusion

This research study has exposed the incidence of aflatoxins contamination in dates varieties (local and imported) collected from the province of Punjab, Pakistan and the incidence rate is high which could lead to creating health threats for consumers. It can be observed that imported varieties also contains higher incidence of aflatoxin contamination which is a clear indicator of poor harvesting, handling, storage and transportation conditions of Pakistan’s vegetable and fruit markets and this provides a gate way for the growth of fungal species. The data from the present study could be used to calculate the risk assessment of Pakistani population and estimate the hazard index for afflicting liver diseases. It is much assumed that with this higher rate of aflatoxin incidence, our population would be at a higher health risk. The current study advised to emphasize a comprehensive and wide-ranging assessment for the contamination of aflatoxins in dates and dates products and strict guidelines should be applied to reduce or evade fungal contamination. This research could also benefit the food authorities for developing standard limits for aflatoxins in dates and dates products of Pakistan because no such limit or standard has been assigned in Pakistan for the same. Furthermore, there is a need to improve the standard agricultural practices and storage facilities of the farms, orchards and warehouses. There is a need to educate the farmers and general population about the health threats of aflatoxins and how to deal with the problem especially during the monsoon season when this issue is at its peak.

References


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