Validation of HPLC Method for the Assessment of Occurrence of Ochratoxin A in Wheat Grains Sold in Major Markets in Lagos

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Abstract:
The presence of Ochratoxin A (OTA) in wheat grain is a serious threat to safety of wheat based foodstuff. In the present study, sixty wheat grain samples were collected from six major markets in Lagos for determination of OTA levels using HPLC-FLD utilizing acetonitrile-water-acetic acid (51:47:2 V/V/V) mixture as mobile phase. The limit of Quantitation (LOQ) was 0.47 µg/kg and the average recovery values of OTA for reference material and spiked wheat grain sample ranged from 87.68-107.09%. Twenty-four (40%) wheat grain samples were contaminated with detectable quantity of OTA ranging from 0.40-41.58µg/kg. OTA levels in five samples (20.8%) exceeded the NAFDAC and European Union (EU) permissible limit of 5.0µg/kg. The expanded uncertainty of measurement calculated signifies that the true value is within ±11.00% of the reported results at a confidence level of 95%. Considering that OTA levels in 20.8% of wheat grain samples analyzed are...
above the permissible limit of 5.0µg/kg, it could be seen as a serious public health threat. Therefore, continuous monitoring and tighter regulation of OTA in cereals is recommended.

**Keywords:** Ochratoxin A, Wheat grain, Method validation, HPLC-FLD, Uncertainty of measurement.

**Introduction**

Wheat (*Triticum aestivum*) grains may become contaminated by molds while in the field and during storage. Some of these molds can produce mycotoxins. Mostly, mycotoxins are stable compounds. The mycotoxins that occur commonly in wheat grains are not destroyed during most food processing operations, which may lead to contamination of finished wheat grain based foods. The mycotoxins that are most common in wheat grains are Ochratoxins and Aflatoxin (Bullerman and Bianchini, 2007). There are three derivatives of Ochratoxin, namely, Ochratoxin A, Ochratoxin B and Ochratoxin C. Ochratoxin A (OTA) is one of the most known and widespread mycotoxins produced by various *Aspergillus* and *Penicillium* species and is classified as a possible human carcinogen (Group 2B) by the International Agency for Research on Cancer (IARC) (WHO, 1994, Elaridi et al., 2019).

Studies have shown that the OTA has carcinogenic, nephrotoxic, immunotoxic, teratogenic, and possibly neurotoxic and genotoxic properties. It has also been associated with Balkan Endemic Nephropathy (BEN) in human, a kidney disease in human that occurred in some areas of Balkan (Pietri et al., 2012, Afshah-Hejri et al., 2013). Human exposure to OTA usually occurs through the consumption of improperly stored food products (Afshah-Hejri et al., 2013). To protect consumers from different health risks, a maximum permissible limits of 5.0µg/kg (5ppb) for OTA in raw wheat and 3.0µg/kg (3ppb) in processed wheat and wheat products was set by EU (European Commission Regulation (EU) No 1881/2006) (EC, 2006a, Alimentarius C. 2009). In Nigeria, wheat is not a major crop; the country imports a significant portion of its wheat to meet domestic demand. However, there is potential to increase production in Nigeria through the implementation of improved seed varieties, modern farming techniques, and better access to finance and markets for farmers. Wheat is a good source of fiber, essential vitamins, and minerals. Eating both refined and unrefined whole wheat is not bad for health but whole wheat is healthier because all the nutrients are intact. A major constraint to wheat grain production and trade is the susceptibility of the crop to fungal invasion especially by fungi which subsequently produce Ochratoxin in grain. The affected wheat grains thus lose their quality and market value due to Ochratoxin contaminations.

Ochratoxins may be present in wheat grains or wheat products even when the mold is not visible and despite the implementation of good agricultural storage and processing practices. Therefore, laboratory analysis for Ochratoxins in wheat and wheat products is necessary. This could be performed by Enzyme-Linked Immuno Sorbent Assay (ELISA), High Performance Liquid Chromatography (HPLC) coupled with a Fluorescence Detector (FLD), or Mass Spectrometry Detector (MSD) (Elaridi et al., 2019). There is paucity of information on OTA status of wheat consumed in Nigeria. Neji et al., (2018) reported on the occurrence of OTA in wheat, Egbontan et al., (2017) also reported a mini survey of mycotoxins including OTA at below detection level in wheat grain. Hence, the aim of the present study is to use HPLC technique for evaluating the occurrence and accurately measure the concentrations of OTA in different wheat grain samples purchased from major markets in Lagos and to validate the method used for analyzing OTA in wheat grains.
Materials And Methods

Reagents and Chemicals

Ochratoxin A analytical standard was obtained from Trilogy Analytical Laboratory (Washington, MO 63090, USA). Ultra-pure water was produced by Elga Water Purification System model PC110COXXMI by Veolia Water Solutions and Technologies, UK. Methanol and Acetonitrile (HPLC – Gradient Grade) were obtained from VWR International Fontenay-Sous-Bois France. Acetic acid was purchased from Merck (Darmstadt, Germany). The OCHRAPREP® (Product Code: P14B) immunoaffinity columns (IAC) was obtained from R-Biopharm Rhone Ltd., Glasgow, Scotland. The IAC has a quoted capacity of ≥ 1200 ng of Ochratoxin A. A reference material (Matrix based multioxins in wheat flour containing 22.0±10.6 µg/kg OTA) was purchased from Trilogy Analytical Laboratory (Washington, MO 63090, USA).

Salts (sodium chloride, disodium hydrogen phosphate, potassium di-hydrogen phosphate and potassium chloride) used in preparing phosphate buffer saline solution (PBS) were all obtained from Merck (Darmstadt, Germany).

Sampling

A total of sixty 60 wheat grain samples were purchased from major markets in Lagos. Ten (10) wheat grain samples were purchased from each major market namely: Alaba, Oshodi, Mushin, Ile-Epo, Oyingbo and Mile-12 respectively. The sampling of the wheat grains was performed as described in Commission Regulation No 401/2006 (EC, 2006b). At least 1 kg of wheat samples were finely ground and all samples were stored at -18°C until the analyses were performed.

Standard Preparation

The Ochratoxin A standard presented as 5.0 mL volume in methanol has a concentration of 9900 ng/mL with an uncertainty value of ±770 ng/mL. The intermediate standard (2475 ng/mL), working standard (0.1 µg/mL) and six point calibrators (0.5 ng/mL, 2 ng/mL, 5 ng/mL, 10 ng/mL, 15 ng/mL and 20 ng/mL) were prepared by dissolving in methanol.

Sample Preparation

A 12.5 g of finely ground test samples were weighed into a 500 mL sample bottle. A 50 mL of 60% acetonitrile was added and was agitated on an orbital shaker at a speed 400 rpm for 10 minutes. The sample was filtered through Whatman No 113 filter paper. 2 mL of the filtrate was diluted with 22 mL of PBS. The diluted filtrate was passed through The OCHRAPREP® IAC without the use of pump allowing it to flow by gravity using manifold set up. The column was washed with 20 mL PBS at a flow rate of 5 mL per minute. Air was passed through the column to remove residual liquid. 1.5 mL acidified methanol (2:98 v/v) was used in eluting the toxin into an amber glass vial from the column at a flow rate of 1 drop per second. Again, 1.5 mL of water was passed through the column and collected into the same vial to give 4 mL total volume. 100 µL was subsequently injected onto the HPLC system (Ochraprep IAC Instruction Manual, 2021)

Instrumentation and Chromatographic Conditions

The reverse phase HPLC analysis was on a Hitachi chromaster system (Hitachi High-Tech Science Corporation, Tokyo, Japan) equipped with a quaternary pump and a Rheodyne injector valve with 100 µL loop. Chromatographic separation was performed on a Chromega Z ECP C18 column (5 µm 100 Å, 25 cm x 4.6 mm) from ES industries, West Berlin, USA. The mobile phase was a mixture of acetonitrile, water and acetic acid (51: 47: 2 v/v/v). Samples were eluted using an isocratic system at a flow rate of 1 ml/min for a run time of 10 min. The analytical column oven was maintained at 40°C. The fluorescence detector (model 5440) was set an excitation and emission wavelength of 333 nm and 443 nm respectively. Injection volume was 100µL. Recording and evaluation of the chromatogram was done with clarity VA chromatography software (version 8.5.0.64, Data Apex Ltd).
Method Validation

The single laboratory validation procedure was used and the analytical performance criteria were assessed in accordance with established guidelines of Food and Drug Administration (FDA, 2019) – Guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics and Veterinary Products and CODEX Alimentarius Commission – Procedural Manual (FAO and WHO, 2019).

Linearity and Range

The linearity of the calibration graph based on the Ochratoxin A solvent standards was established over the concentration range of 0.5 – 20 µg/kg with a correlation coefficient ($r^2$) of 0.995839 (fig 1). The retention time was quiet stable with variable across various batches within ±0.2 min (fig 2 and fig 3 for standards overlay and positive sample). Additionally, the residual of each calibration levels were calculated and they all fall with the allowable range of ±20%.

Figure 1. Calibration for Ochratoxin a Standard Range from 0.5 – 20 µg/kg

Figure 2. OTA Standard Levels Overlay Showing a Retention Time within a Window of ±0.2 min
Precision and Trueness

The precision was established by analyzing a blank wheat sample spiked with 0.5, 10 and 20 ng/ml of OTA standard. The samples were analyzed in six replicates for repeatability and thirty replicates within eight days for intermediate precision and the RSD was calculated. The results are provided in Tables 1 and 2. The method trueness was investigated using the recovery of the reference material and spiked samples. The precision in this study as adjudged by the relative standard deviation in the range of 0.8% – 6.2% is below the stated criteria of 22% maximum. The intermediate precisions of the reference material and spiked sample were within the acceptable range as shown in table IV. The recovery for this study was assessed using the reference material and spiked samples. The result for the recovery of reference material (86.0%) and the spiked sample (87.68% - 107.09%) falls within the acceptable recovery range as stated in the CODEX /FDA guidelines. The result as shown in tables 1 and 2 demonstrate the fitness of the method as stated in FDA/CODEX guidelines.

Limit of Detection (LoD) and Limit of Quantitation (LoQ)

The visual evaluation (empirical method) was used to determine the detection and quantitation limit. The spiked concentration was steadily decreased until the minimum level at which the analyte could be reliably detected. This spiked level was at 0.25µg/kg and the average of 20 analysis result was used to calculate the LoD and LoQ using the formula below

\[
\text{LoD} = 3 \times \text{SD} + C_{\text{ave}}
\]
\[
\text{LoQ} = 10 \times \text{SD} + C_{\text{ave}}
\]

SD: standard deviation of measurement

C_{\text{ave}}: average concentration of spike sample

0.32 µg/kg and 0.47 µg/kg were obtained as the LoD and LoQ respectively.

The Codex and FDA guidelines stated that when the maximum permissible level of a contaminant is below 100 µg/kg, the LoD and LoQ should not be more than one fifth (1/5) and two fifth (2/5) of the specified maximum permissible level respectively. The maximum permissible level for ochratoxins is 5.0 µg/kg. From the results obtained in this study, the LoD and LoQ
(0.32 µg/kg and 0.47 µg/kg respectively) meet the criteria.

Table 1. Precision RSD (Within-day) and Recovery of Spiked and Reference Material (n = 6)

<table>
<thead>
<tr>
<th>Reference Material</th>
<th>Replicate Analysis of OTA (µg/kg)</th>
<th>Average (µg/kg)</th>
<th>% Recovery</th>
<th>SD (µg/kg)</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat Reference Material</td>
<td>18.804 19.051 18.977 18.690 19.012 18.990</td>
<td>18.921</td>
<td>86.003</td>
<td>0.14</td>
<td>0.748</td>
</tr>
<tr>
<td>Spiking Level (µg/kg)</td>
<td>0.5 0.482 0.503 0.518 0.504 0.458</td>
<td>0.502</td>
<td>100.5</td>
<td>0.031</td>
<td>6.229</td>
</tr>
<tr>
<td>20</td>
<td>17.546 17.758 17.614 17.321 17.460 17.513</td>
<td>17.535</td>
<td>87.677</td>
<td>0.147</td>
<td>0.838</td>
</tr>
</tbody>
</table>

Table 2. Intermediate Precision RSD (Between-day)

<table>
<thead>
<tr>
<th>Reference Material</th>
<th>Average (µg/kg)</th>
<th>SD (µg/kg)</th>
<th>RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat Reference Material</td>
<td>17.80</td>
<td>1.09</td>
<td>6.15</td>
</tr>
<tr>
<td>Spiking Levels (µg/kg)</td>
<td>0.5 0.51</td>
<td>0.03</td>
<td>6.54</td>
</tr>
<tr>
<td>10</td>
<td>10.63</td>
<td>0.29</td>
<td>2.75</td>
</tr>
<tr>
<td>20</td>
<td>17.82</td>
<td>0.52</td>
<td>2.89</td>
</tr>
</tbody>
</table>

Using the NORDEST approach (Magnusson et al., 2012), the measurement uncertainty of the method was calculated from result of replicate analysis of the control sample (within laboratory reproducibility or intermediate precision) for random error as well as data from method validation (recovery from spiking) for systematic error. These two were combined to obtain the combine uncertainty (uc) and multiplied by a coverage factor of 2 to give expended uncertainty (Uc) providing a confidence level of 95%. The formulæ are shown in Table 3. The Expanded uncertainty obtained was ±11.00%. This value signifies that the true value is within ±11.00% of the reported result value, 95% of the time.

Table 3. Uncertainty Components and Their Formulae

<table>
<thead>
<tr>
<th>Uncertainty component</th>
<th>Definition</th>
<th>Formulae</th>
</tr>
</thead>
<tbody>
<tr>
<td>uc</td>
<td>Combined standard uncertainty</td>
<td>√(u(Rw))² + (u(bias))²</td>
</tr>
<tr>
<td>u(Rw)</td>
<td>Standard uncertainty component for the within laboratory reproducibility</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>u(bias)</td>
<td>Uncertainty component associated with the possible method and laboratory bias</td>
<td>√(bias)² + (√bias)² + u(recovery)²</td>
</tr>
<tr>
<td>u(recovery)</td>
<td>=√(u(conc))² + u(vol)²</td>
<td></td>
</tr>
<tr>
<td>u(conc.)</td>
<td>= Certificate of Analysis</td>
<td></td>
</tr>
<tr>
<td>u(vol.)</td>
<td>= (repeatability and temperature effect u(v, temp))</td>
<td></td>
</tr>
<tr>
<td>u(v, temp)</td>
<td>= √(ΔT)² + $\sqrt{\frac{\Delta \beta}{\beta}}$</td>
<td></td>
</tr>
<tr>
<td>Uc</td>
<td>Expanded uncertainty at approximately 95% confidence interval</td>
<td>2 x uc</td>
</tr>
</tbody>
</table>
Table 4. Occurrence and Concentration Levels of OTA in Wheat Grain Samples Sold in Major Markets in Lagos

<table>
<thead>
<tr>
<th>Location</th>
<th>No of Analyzed Samples</th>
<th>No of Positive Samples</th>
<th>% of Positive Sample</th>
<th>No of Positive Sample &gt;5.0µg/kg</th>
<th>Conc. Range of OTA µg/kg</th>
<th>Mean Conc. of OTA µg/kg ± Uncertainty*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alaba</td>
<td>10</td>
<td>5</td>
<td>50</td>
<td>1</td>
<td>0.58-5.73</td>
<td>1.28±0.14</td>
</tr>
<tr>
<td>Oshodi</td>
<td>10</td>
<td>2</td>
<td>20</td>
<td>1</td>
<td>0.52-1.22</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>Mushin</td>
<td>10</td>
<td>2</td>
<td>20</td>
<td>1</td>
<td>1.44-26.47</td>
<td>3.10±0.34</td>
</tr>
<tr>
<td>Ile-Epo</td>
<td>10</td>
<td>4</td>
<td>40</td>
<td>2</td>
<td>0.49-7.53</td>
<td>1.54±0.17</td>
</tr>
<tr>
<td>Oyingbo</td>
<td>10</td>
<td>9</td>
<td>90</td>
<td>1</td>
<td>0.40-2.21</td>
<td>4.21±0.46</td>
</tr>
<tr>
<td>Mile-12</td>
<td>10</td>
<td>2</td>
<td>20</td>
<td>1</td>
<td>0.54-41.58</td>
<td>1.91±0.21</td>
</tr>
<tr>
<td>TOTAL</td>
<td>60</td>
<td>24</td>
<td>40</td>
<td>5</td>
<td>0.40-41.58</td>
<td></td>
</tr>
</tbody>
</table>

Note: the uncertainty value signifies that the result is within the stated range at a confidence level of 95%

Result and Discussion

Table 4 summarizes the incidence and concentration of Ochratoxin A (OTA) in wheat grain samples from six major markets in Lagos, Nigeria. Out of the sixty wheat grain samples analyzed, twenty-four samples were found to be contaminated with OTA corresponding to 40% at varying concentration of 0.40-41.58µg/kg. While the remaining thirty-six samples (60%) were below the detection limit of the method (0.32µg/kg). Results of our study showed that five (20.8%) wheat grain samples out of the twenty-four contaminated with OTA were having concentration greater than 5.0µg/kg limit established for unprocessed cereals by NAFDAC and EU (Commision Regulation EU No 1881/2006). The trend of concentrations of OTA greater than the established limit of 5.0µg/kg in this study is shown in a decreasing order of 41.58µg/kg (Mile-12 market) > 26.47µg/kg (Mushin Market) > 7.53µg/kg (Ile-epo Market) > 5.81µg/kg (Ile-epo Market) > 5.73µg/kg (Alaba Market). The remaining nineteen contaminated wheat grain samples corresponding to 79.2% have concentrations less than 5.0µg/kg. Hence, these samples do not pose any health challenge. The variations in OTA levels in wheat grain samples from different markets of Lagos may be attributed to fungal growth mainly influenced by climate condition viz. temperature, humidity and poor agricultural practice such as inadequate aeration or long-term storage without adequate drying.

Atumo.S (2020) corroborated this rationale stating biotic factors namely water availability; temperature; gas composition and moisture content are responsible for the formation of OTA on wheat grain samples. Joubrane et al., (2020) alluded that insect infestation can cause damage to the grain tegument, resulting in production of carbonic acid and water, this process contributes to an increase in humidity, which in turn can cause an increase in grain respiration and consequential increase in temperature. Under these conditions, there is a serious risk of mold growth that could lead to the production of OTA.

Studies related to contamination of wheat grains and other cereals with OTA have been reported from various part of the world. For instance, in Nigeria, Neji et al., (2018) detected OTA in 5 wheat samples at a level ranging from 0.42 – 0.44 µg/kg with a mean value of 0.43 µg/kg using HPLC-FLD. The frequency of occurrence of OTA in wheat grain samples obtained from present study - 24 of 60 (40%) similar to the study of Nida and Ahmed (2010) who reported the presence of OTA in 5 of 17 (29%) wheat samples in Jordan. The slight difference in frequency of occurrence (40% as against 29%) may be attributed to climate and possibly better agricultural practices that the latter adheres to.

In another related study, Darwish et al., (2014) reported that OTA occurred in 24.3% of wheat, sorghum and barley samples in Ethiopia at a mean concentration of 54.1µg/kg and a
maximum of 2,106µg/kg. El-Naggar et al. (2018) reported that Ukrainian wheat samples had OTA content ranging from 0.5-1.367µg/kg with an average of 1.047µg/kg. The result is lower compared to this current study which has a concentration range of 0.41-41.58µg/kg and overall mean of 1.91µg/kg. The results obtained in this study is similar to Kumar et al. (2012) who found OTA in wheat samples from India with a contamination range 1.36-21.17µg/kg including 13(26%) samples exceeding the 5.0 µg/kg established limit EU.

High concentration of OTA in some of the wheat grain samples reported in this study can promote the occurrence of renal diseases and immune suppression when people and animal consume the contaminated samples.

Conclusion

This current study employed the HPLC-FLD method to determine OTA levels in wheat grains. The method was found to be fit for purpose as all the method performance characteristics were within the acceptable criteria set by CODEX and FDA. A total of sixty samples of wheat grains from different markets in Lagos were examined. 24(40%) of the samples contained OTA at detectable levels. Five (5) out of the 24 (20.8%) showed contamination of OTA above the established limit of NAFDAC and EU (5.0µg/kg). However, this must not be underestimated as they may occur in other wheat grain samples within the same market. It is therefore, pertinent to necessitate the analysis of several grain samples for OTA periodically in order to ascertain the likely degree of human exposure to this hazardous substance.

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