Determination of Aflatoxins (B1, B2, G1 and G2) in milled cereals and grains by HPLC-FLD

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1. **Introduction**

Contamination of foodstuffs with mycotoxins is one of the most concerning problems in food and feed safety globally. Mycotoxins are secondary metabolites produced by filamentous fungi, having toxic and destructive biological effects on animal and human tissues. A group of toxins produced by the common mould Aspergillus flavus is known as aflatoxins. Based on their fluorescent property they are named as B1, B2 (exhibit blue fluorescence) and G1, G2 (exhibit green fluorescence). The hydroxy toxins M1 & M2 produced by cattle after feeding on contaminated feeds are also known as milk toxins.

Aspergillus flavus thrives mainly on nuts, oil seeds, cereals, pulses and dried fruits. Wet and humid pre-harvest conditions as well as improper post-harvest storage of foods are conducive for the growth and proliferation of the fungi.

Moisture content of above 9%, high humidity and temperatures above 30°C are conducive for the production of aflatoxins.

The fluorescent property of aflatoxins is widely used for their detection and determination in foods. Immuno-affinity cleanup methods have been found to be very effective in the selective cleanup of toxins from other interfering materials.

HPLC separation followed by post-column derivatization of the toxins has been found to improve the fluorescence of B1 and G1 without affecting the fluorescence of B2 and G2 which makes the technique very sensitive and suitable for the determination of aflatoxins.

2. **Scope and objective**

This method is applicable for the determination of aflatoxins (B1, B2, G1 and G2) in ppb levels in maize grains, peanut and milled cereal samples.

3. **Abbreviations and definitions**

   ppb: parts per billion  
   AR: analytical reagent  
   HPLC: High-Performance Liquid Chromatography  
   rpm: revolutions per minute  
   PTFE: Polytetrafluoroethylene  
   AFB1: aflatoxin B1  
   AFB2: aflatoxin B2  
   AFG1: aflatoxin G1  
   AFG2: aflatoxin G2

4. **Principle of the method**
Aflatoxins are extracted, purified, separated by reverse phase liquid chromatography and then derivatised i.e UVETM - Photochemical Post Column Derivatization and then and detected by fluorescence.

5. **Safety considerations and precautions**
The laboratory is a high risk area, and therefore proper care shall be taken while handling the chemicals and reagents etc. Use proper protective equipment and wear lab coats. Use safety glasses for eye protection, and gloves to protect the skin from toxic and corrosive materials. Use respirators if adequate ventilation is not available.

6. **Materials**

6.1. **Reagents and solvents**
- Water (HPLC grade)
- Sodium chloride (AR grade)
- Methanol (HPLC grade)

6.2. **Equipment, instruments and consumables**
- Beaker
- Micro pipettes
- Quartz cuvettes
- UV –Visible Spectrophotometer
- Liquid chromatography- HPLC with a Fluorescence detector
- Analytical balance
- 0.45 μm filters
- Aflatoxin immunoaffinity columns
- Volumetric flasks
- Analytical balance
- Ultra turax homogenizer
- Vortex mixer
- Reversed phase C-18 column (particle size 5 μm, 250 × 4.6 mm or equivalent)
- Membrane filter (0.45 μm)
- Ultrasonic bath
- Reagent bottle
- Falcon tubes
- Filter paper, qualitative No. 4 or equivalent
- Glass microfiber filter papers
- PTFE filter
- Autosampler vials
- Glass tubes
- Photochemical Post-Column Derivatization
- Micropipette 5 μL – 100 μL, adjustable
- Vacuum manifold
7. Solutions
7.1 Preparation of mobile phase, methanol/water (70/30).

Prepare mobile phase by volume ratio.
- Measure 700 ml of methanol in a measuring cylinder.
- Measure 300 ml of distilled water in a measuring cylinder.
- Mix 1 and 2 thoroughly and degas

7.2 Preparation of mobile phases
- Mobile phase A: Measure 1000ml of methanol (HPLC Grade) into the mobile phase bottle and place in ultrasonic bath for 15 minutes.
- Mobile phase B: Measure 1000ml of Water (HPLC Grade) into the mobile phase bottle and place in ultrasonic bath for 15 minutes.

8. Standards
8.1. Standard substances
Aflatoxin B1, B2, G1 and G2 standards (Sigma or equivalent) with stated concentrations of each component e.g. 20µg/ml of each component and purity of ≥ 98 of the component.

8.1.1. Preparation of individual standard stock solution:
Aflatoxin standards are received in minimal quantity (with exact amount mentioned) in amber coloured containers. Hence, for preparing a required concentration, appropriate volume of solvent is directly added to the bottle and stock solution is prepared. Calculate the Concentration of the standard in µg/mL.

- Weight of standards (corrected, a) = [weight of standards × Purity]/100
- Volume of solvent (mL, b) = [Weight of solvent / specific gravity]
- Calculated Conc. (µg/mL) =
  [Corrected weight of standard (a) / volume of solvent (b)] x 1000
- Label the container with lab code, name of chemical, conc.in µg/mL, name of solvent, date of presentation, date of expiry.
- Enter the data in the respective log book and registers.

8.1.2. Preparation of intermediate standard stock solutions:
- Before use, the stock solution is sonicated and vortexed thoroughly.
- From the above standard stock, using dilution process and the formula C1V1=C2V2,
  a intermediate standards solution concentration 4µg/mL of individual aflatoxins prepared in required volume.

8.1.3. Analysis by UV-Spectrophotometer:
- Since aflatoxins are photosensitive and get degraded upon exposure to light during storage, their accurate concentration is determined by UV-Spectroscopy.
• Since these individual aflatoxins contain chromophore groups, which can absorb light of a particular wavelength, they can be detected and qualified using Beer Lambert’s law.
• After switching on the spectrophotometer, wait for initialization of instrument.
• Initially, baseline corrections are performed specifying the range for measurement viz. 200 to 800 nm absorbance as 0 to 1.
• Using UV-spectrum mode, lambda max (\( \lambda_{\text{max}} \)) was identified respectively for each aflatoxin for the range described above.
• Further, the absorbance value was measured at \( \lambda_{\text{max}} \) in equation mode.
• The absorbance value was used to calculate the actual concentration from a formula derived from Beer Lambert’s law, as follows:

\[
A = \varepsilon l c \\
A = \text{absorbance} \\
\varepsilon = \text{absorptivity} \\
l = \text{pathlength} \\
c = \text{concentration}
\]

Absorbance = molar absorptivity x path length of the cuvette (1 cm) x concentration
Concentration (mg/ml)=\( \frac{A \times \text{Mol.wt} \times 1000}{(\text{molar absorptivity} \times \text{path length})} \)
• This is considered as correct concentration which is applied for preparation of working standard solution mixture and calibration standards.

8.1.4 Preparation of working standard solution mixture:
• Similarly, a working standard solutions mixture (100ppb) is prepared from individual intermediate standard stock solution (4ppm) by dilution and use of C1V1=C2V2 formula.
• Application of correction factor to the concentration of individual intermediate standard stock solution will assure correct preparation of further mixture and dilutions.

8.2 Stock solution (1000 mg/L)
Dissolve 10 mg each standard aflatoxin B1, B2, G1, G2 in methanol and make up to 10 mL in a volumetric flask. If required, determine their purity by Molar extinction coefficient method following the procedure described in AOAC.

8.3 Intermediate mixed stock solutions preparation (1 mg/L)
Take 100 \( \mu \)L of each aflatoxin stock solution (1000 mg/L) into 100 mL volumetric flask and dilute with methanol.
NB: Already prepared aflatoxin mixes of 1mg/L from reputable manufacturers can be used as long as they are certified reference material with certificate of analysis containing measurement uncertainty and coverage factor and traceable.

8.4. Working standard
Prepare working standard at 4 µg/L of by taking 40 µL of 1 mg/L solution into 10 mL volumetric flask and make to the mark with methanol/water (50/50, v/v).

8.5. Calibration standards

<table>
<thead>
<tr>
<th>Stock concentration (µg/L)</th>
<th>Volume of stock (mL)</th>
<th>Volume dilution</th>
<th>Actual concentration (µg/L)</th>
<th>Method factor</th>
<th>Method Conc. (µg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>2</td>
<td>-</td>
<td>4.0</td>
<td>8</td>
<td>32</td>
</tr>
<tr>
<td>4.0</td>
<td>2</td>
<td>2</td>
<td>2.0</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
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<td>2</td>
<td>1.0</td>
<td>8</td>
<td>8</td>
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<tr>
<td>1.0</td>
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<td>0.5</td>
<td>8</td>
<td>4</td>
</tr>
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<td>2</td>
<td>0.25</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>0.25</td>
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<td>2</td>
<td>0.125</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>0.125</td>
<td>2</td>
<td>2</td>
<td>0.0625</td>
<td>8</td>
<td>0.5</td>
</tr>
<tr>
<td>0.0625</td>
<td>2</td>
<td>2</td>
<td>0.03125</td>
<td>8</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Note
From the table, standards equivalent 0.25, 4, 8, 16 and 32 µg/kg are used for calibration.

9. Procedure
9.1.1. Sample preparation

Nuts and grains
Grind entire quantity of sample (2–5 kg) using romer laboratory mill or equivalent. Mix thoroughly to get a well homogenized sample. Take 200g of homogenized sample and grind to fine particle size with a bench mill.

Peanut butter
Transfer the entire sample from bottles into a dry vessel. Mix well to get a homogenized sample.

Milled cereals
Mix thoroughly to get a well-homogenized sample.

Drilled feeds and samples
Grind the sample if necessary, using romer laboratory mill or equivalent, and mix thoroughly to get a well-homogenized sample.

9.1.2. Quality control samples
At least one quality check shall be included with each batch of analysis, and results should be entered into controls chars to monitor the trend.

The analyst shall prepare an additional control sample by spiking a blank sample at 5 µg/kg using a different batch of the stock standard not used for creating a calibration curve and leave in the fridge overnight after spiking for use the following day or running rest portion of Proficiency Testing sample of known concentration and analyzing it alongside each batch of samples.

**9.1.3 Reagent blank (RB)**

RB shall contain the same volumes of all reagents used in the processing of the samples. The reagent blank shall be carried through complete procedure and contain the same acid concentration in the final solution as the sample solution used for analysis. Reagent Blank (RB) shall be prepared at the same time and under the same conditions.

**9.1.4 Sample extraction**

Weigh 20 ± 0.0010 g of sample in reagent bottle and add 4 g of sodium chloride. Add 100 mL of methanol/water (70/30) and blend with Ultra turax at 25,000 rpm for 3 min. Filter the extract using a qualitative filter paper into a clean 50 mL Falcon tube. Pipette 7.5 mL of the extract into a clean 50 mL falcon tube. Dilute extract with 15 mL of distilled water and vortex for 1 min. Filter the dilute extract through a glass microfibre filter into a clean 50 mL falcon tube (if filtrate is still turbid, vortex for 5 min at maximum speed).

**Note**

If a microfibre filter is not available, vortex for 5 min at maximum speed, centrifuge at 5000rpm for 10 minutes and filter through normal qualitative filter paper.

**9.1.5 Sample cleanup**

Remove two end caps of immunoaffinity column (Aflatest or equivalent). Attach the column to a reservoir syringe and place into a vacuum manifold. Pass 7.5 mL of filtered diluted extract (equivalent to 0.5 g of sample) through Aflatest column at a rate of about 1–2 drop/s until air comes through the column. Next, pass 10 mL of distilled water through the column at a rate of about 2 drop/s. Repeat the previous step with another 10 mL of distilled water. Remove excess water in the system by suction (Make sure the columns do not dry). Place glass tubes into the manifold. Add 1 mL of methanol into the immunoaffinity column. Allow three to four drops of methanol into the glass tubes, and then close the taps of the manifold. Allow methanol to soak for 5 min. Then elute the column at a rate of 1–2 drop/s into the glass tubes. Add another 1 mL of methanol and elute the column same way after allowing methanol to soak for 5 min. Remove excess methanol by suction. Remove the glass tubes from the manifold and add 2 mL of HPLC water. Cap and vortex for 1 min and filter with 0.45 µm PTFE filter into auto sampler vial, ready for HPLC analysis.

**10 Instrumental analysis (HPLC-FLD)**

Analysis of aflatoxins is carried out using an HPLC instrument with a fluorescence detector. Conditions of HPLC analysis are given below.

- Separation mode: reversed-phase chromatography
• Analytical column: L 5 RPC-18 (4.6 x 250 mm, particle size 5 µm) or equivalent
• Column temperature: 40°C
• Post column reactor coil temperature: 40°C
• Injection volume: 40 µL
• Flow rate: 0.65 mL/min
• Run time: 20 Minutes
• Mobile phase: A= Methanol, B=Water
• A=50%, B=50%, Mobile phase: Methanol/water (50/50)
• Elution mode: Isocratic
• Fluorescence detector settings: 362 nm (excitation), 455 nm (emission)

9 Calculation and interpretation of results

\[
\text{Aflatoxin, ppb} = \frac{C \times D \times 20}{W}
\]

Where:
- **D** = Dilution factor, If sample is diluted further
- **W** = Weight of sample in grams.
- **20** = Factor catering for the weight of sample if more or less of sample is weighed
- **W** = Weight of sample
- **C** = Concentration of Standard (µg/kg).

10 Method validation

The precision (repeatability and reproducibility) and accuracy of the method was determined using rest portion of proficiency testing sample.

<table>
<thead>
<tr>
<th></th>
<th>% Accuracy</th>
<th>Linearity</th>
<th>LOD</th>
<th>LOQ</th>
<th>Working range (ppm)</th>
<th>%RSD repeatability</th>
<th>%RSD reproducibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2</td>
<td>88.5</td>
<td>r²=0.99999</td>
<td>0.06</td>
<td>0.25</td>
<td>0.25-32.0</td>
<td>5.2</td>
<td>5.2</td>
</tr>
<tr>
<td>G1</td>
<td>85.9</td>
<td>r²=0.99999</td>
<td>0.125</td>
<td>0.25</td>
<td>0.25-32.0</td>
<td>10.1</td>
<td>9.9</td>
</tr>
<tr>
<td>B2</td>
<td>79.8</td>
<td>r²=0.99999</td>
<td>0.060</td>
<td>0.25</td>
<td>0.25-32.0</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>B1</td>
<td>81.8</td>
<td>r²=0.99998</td>
<td>0.125</td>
<td>0.25</td>
<td>0.25-32.0</td>
<td>3.2</td>
<td>3.2</td>
</tr>
</tbody>
</table>

11 References

11.1 (Ref :- A.O.A.C 17th edn, 2000, Official Method 977.16 Sampling of Aflatoxins, Preparation of Sample)
11.2 AOAC Official Method 991.31 for the determination of aflatoxins (AFs; sum of aflatoxins B1, B2, G1, and G2) in corn, raw peanuts, and peanut butter by using immunoaffinity column cleanup with LC

11.3 Aflatest Instruction Manual
Schematic diagram of extraction procedure

Sample extraction

1. Weigh 20 ± 0.0010 g of sample in a reagent bottle
2. Add 4 g of NaCl
3. Add 100 mL of methanol/water (70/30)
4. Blend with ultra turrax at 25,000 rpm for 3 min
5. Filter extract using a qualitative filter paper into a 50 mL falcon tube.
6. Pipette 7.5 mL of extract into a 50 mL falcon tube.
7. Dilute the extract with 15 mL of water.
8. Vortex for 1 min.
9. Filter the diluted extract through a glass microfiber filter in to a 50 mL falcon tube.

Sample clean up

1. Pass 7.5 mL of filtered diluted extract through Aflatest column (1–2 drop/s) until air comes through the column.
2. Pass 10 mL of distilled water (2 drop/s)
3. Remove excess water by suction.
4. Pass another 10 mL of distilled water (2 drop/s) and remove excess water by suction.
5. Place glass tubes into the manifold.
6. Add 1 mL methanol to the immunoaffinity column.
7. Allow 3–4 drops of methanol into the glass tubes and close the taps of the manifold.
8. Allow methanol to soak for 5 min.
9. Elute the column into the glass tube (1–2 drop/s)
10. Add another 1 mL of methanol and repeat steps 7, 8 and 9.
11. Remove excess methanol by suction.
12. Add 2 mL of water to eluate.
13. Vortex for 1 min.
14. Filter with 0.45 µm PTFE filter into autosampler vial.