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GUIDELINE FOR SAMPLING AND TESTING FOR AFLATOXIN

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1. INTRODUCTION

Objective of the User Guide

This user guide was created to assist farmers, traders, processors and certified graders to understand and apply the correct procedure in determination of aflatoxins in grains. It aims at ensuring that farmers, traders, grain handlers, and processors meet the maximum aflatoxin levels, reduce post-harvest wastage and provide safe, appropriate quality grain to consumers.

Basis of the User Guide

- This is a user guide for anyone working on the grain quality analysis.
- This guide outlines the procedures for sample preparation.
- This user guide can be adapted to train graders or grain inspectors on determination of aflatoxins before grading of grain.

2. ABOUT AFLATOXINS

Mycotoxins are in the family of secondary metabolites of molds. They contaminate food and feed grains during the pre- and post-harvest period. The contamination is transferred throughout the primary and secondary processing, resulting in food and feed commodities capable of transmitting toxins to humans and livestock. Molds proliferate in high moisture ambient environments, are ubiquitous in nature and therefore they are ever present in the environment. Therefore, systematic management of mold is pivotal to a sustainable, healthy flow of grains to the food and feed industries.

Aflatoxins is a type of Mycotoxins that are produced by some species of fungi in the genus Aspergillus, the most notable being Aspergillus flavus and A. parasiticus. These fungi invade crops during maturation in the field and during storage contaminating them with aflatoxins. Maize is one of the most susceptible cereal crops to pre- harvest aflatoxins contamination particularly during periods of moisture stress and when insect damage is prevalent. Post-harvest contamination in maize also occurs when grains are stored in damp environments. The Food and Agriculture Organization estimates that Aflatoxins contaminate about 25% of agricultural crops worldwide.

The aflatoxins can grow in the field before harvest and in storage conditions between 20 and 40°C with a 10- 20% of humidity, and 70-90% of relative humidity in the air. Post -harvest contamination can occur if crop drying is delayed and during storage of the crop if moisture is allowed to exceed maximum values. Aflatoxin contamination is also promoted by stress or damage to the crop due to drought before harvest, the insect activity, a poor timing of harvest, the heavy rains during and after harvest, and an inadequate drying of the crop before storage.

3. DETERMINATION OF AFLATOXIN B1, B2, G1 AND G2

> **Representative samples have to be taken to a laboratory capable of testing for Aflatoxin.**

The presence of Aflatoxins is tested by thin layer chromatography, High performance Liquid Chromatography (HPLC) or absorbance meters e.g. ELISA readers. Representative samples have to be taken to a laboratory that is capable of carrying out Aflatoxin test.

3.1. PRINCIPLE

The test sample is extracted with a mixture of methanol and water. The sample extract is filtered and diluted with buffer or water and applied to an affinity column containing antibodies specific for aflatoxins B1, B2, G1 and G2. The aflatoxins are isolated, purified and concentrated on the column then removed from the antibodies with methanol. The aflatoxins are quantified by reverse–phase High Performance Liquid Chromatography (HPLC) with fluorescence detection and post column derivatization.

3.2. APPARATUS

3.2.1. Immuno affinity (IA) column, which contains antibodies raised against aflatoxin B1, B2, G1 and G2. The column shall have a minimum binding capacity of not less than 100 ng of aflatoxin B1. It shall give a recovery of not less than 80 % for aflatoxin B1, B2, G1, and not less than 60 % for aflatoxin G2, when a standard solution in 15 ml of a methanol/water mixture [1 part methanol and 3.4 parts water (by volume)] containing 5 ng of each toxin is applied to the IA column. The IA column should be equipped with an appropriate solvent reservoir (for example, a syringe with adapter). It is advisable to carry out recovery experiments for every matrix that the method is used for.

3.2.2. Blender, the use of a high-speed blender is recommended.

3.2.3. Fluted filter paper, for example, 24-cm diameter.

3.2.4. Glass microfibre filter paper for example, 11-cm diameter. For example, Whatman 934AH is appropriate for this purpose. Other products may be used if they can be shown to give comparable results.

3.2.5. Volumetric flasks, Class A grade, of 2-ml capacity.

3.2.6. Spectrometer, capable measuring wavelengths between 200 nm and 400 nm.

3.2.7. Quartz glass cells, of optical path length 1 cm, and with no significant absorption between wavelengths of 300 nm and 370 nm.

3.2.8. Membrane filter for aqueous solutions, made of polytetrafluoroethylene (PTFE), with a diameter of 4 mm and a pore size of 0.45 µm.

3.2.9. HPLC apparatus, comprising the components in 3.2.10 to 3.2.12

3.2.10. HPLC pump, capable of producing a flow rate at 1 ml/min

3.2.11. Injection system, a syringe-loading injection valve with 50-µl loop or equivalent

3.2.12. Analytical reverse-phase separating column, for example, C18, which ensures a baseline resolved resolution of the aflatoxin B1, B2, G1 and G2 peaks from all other peaks, with the following characteristics:

a) length of 250 mm;
b) internal diameter of 4.6 mm; and
c) spherical particle size of 5 µm.

Shorter columns may be used.

3.2.13. Post-column derivatization system, consisting of a pulse-free pump and very low dead- volume T- piece, with polytetrafluoroethylene (PTFE) or stainless-steel tubing of length 3 000 mm to 5 000 mm and internal diameter of 0.5 mm, and a heating bath or post-column reactor for the iodine reaction

3.2.14. Fluorescence detector, with excitation at wavelength of 365 nm and emission at wavelength of 435 nm (for filter instruments: emission wavelength at 400 nm), capable of detecting at least 0.05 ng of aflatoxin B1 per injection volume (here 50 µl).

4. REAGENTS

Use only reagents recognized analytical grade, unless otherwise stated.

4.1. Water, according to grade 1 of ISO 3696

4.2. Sodium chloride.

4.3. Iodine, crystalline.

4.4. Aflatoxin, in crystal form or as a film ampoule

WARNING
Aflatoxins are carcinogenic to human subjects. Adequately protect from daylight the laboratory where the analyses are carried out. This may be achieved effectively by using ultraviolet (UV) absorbing foil on the windows in combination with subdued light (no direct sunlight), or curtains or blinds in combination with artificial light (fluorescent tubes are acceptable).

4.5. Acetonitrile, HPLC grade

4.6. Methanol, analytical grade 4.7. Methanol, HPLC grade

4.8. Toluene, analytical grade

WARNING
Toluene is highly flammable and harmful. Standard preparation involving this solvent shall be performed in a fume cupboard. Operations outside the fume cupboard, such as measurement of standards by UV spectrometry, shall be performed with the standards in closed containers.

4.9. Toluene/acetonitrile mixture. Mix 98 parts per volume of toluene (4.8) with 2 parts per volume of acetonitrile (4.5). [See Warning in 4.8].

4.10. Extraction solvent. Mix 7 parts per volume of methanol (4.7) with 3 parts per volume of water (4.1).

4.11. Mobile phase. Mix 3 parts per volume of water (4.1) with 1 part per volume of acetonitrile (4.5) and 1 part per volume of methanol (4.7). Degas the solution before use.

4.12. Post-column derivatization reagent. Dissolve 100 mg of iodine (4.3) in 2 ml of methanol (4.8). Add 200 ml of water (4.1), stir for 1 h, then filter through a 0.45-µm membrane filter (3.2.8). Prepare the solution the week of use and store the solution in the dark or in a brown glass bottle. Before use, stir the solution for 10 min.

5. AFLATOXIN B_1, B_2, G_1 AND G_2 STOCK SOLUTIONS

WARNING
Protect solutions containing aflatoxin from light as far as possible (keep in the dark, use aluminium foil or amber-coloured glassware).

Dissolve aflatoxin B1, B2, G1 and G2 separately in the toluene/acetonitrile mixture (4.9) to give separate solutions containing 10 µg/ml.

To determine the exact concentration of aflatoxin in each stock solution, record the absorption curve at a wavelength between 330 nm and 370 nm in 1-cm quartz glass cells (3.2.7) using a spectrometer (3.2.6) with a toluene/acetonitrile mixture (4.9) as reference.

Calculate the aflatoxin concentration of each aflatoxin, þi, in micrograms per millilitre, using **Equation** (1) below.

$$\text{þi} = \frac{A_{max} \times M_i \times 1000}{\varepsilon_i \times d}$$

where

A_{max} is the absorbance determined at the maximum of the absorption curve;

M_i is the molecular mass, in grams, of each aflatoxin; and

ε_i is the molar absorption coefficient of each aflatoxin in toluene/acetonitrile;

d is the optical path length, in centimetres, of the cell.

NOTE: This value is determined in a solution that contains c = 1 mol/l of aflatoxin and in a cell with the optical path length d = 1 cm. The molar absorption coefficient ( ) is usually given without a unit of measurement, but from the equation A = x c x d, the following unit can be derived for it: 1 mol-1 cm-1. Mi and i are given in Table 1.

Aflatoxin	*M* i	i
B1	312	19,300
B2	314	20,400
G1	328	16,600
G2	330	17,900

Table 1: Molecular mass and molar absorption coefficient of aflatoxins B1, B2, G1 and G2

5.1. Stock solution of mixed aflatoxins. Prepare a stock solution containing 500 ng/ml of aflatoxin B1, 125 ng/ml of aflatoxin B2, 250 ng/ml of aflatoxin G1 and 125 ng/ml of aflatoxin G2 in toluene/acetonitrile (4

5.2 .9). If the solution has to be stored, weigh the flask before storage. Wrap the flask tightly in aluminium foil and store it at approximately 4 °C. Immediately before use, reweigh the flask and record any change in mass after storage.

NOTE: Normal exposure to UV light during absorbance measurement results in no observable conversion to photoproducts.

5.3 Standard solution of mixed aflatoxins

- Transfer each quantity, as specified in Table 2, of mixed aflatoxin stock solution (5.1) into a series of four 2-ml volumetric flasks (4.5).
- Evaporate the solutions just to dryness under a stream of nitrogen at room temperature.
- To each flask, add 1 ml of methanol (4.6). Dissolve the dry residue in it, dilute the solution to the mark with water (4.1) and mix.
- Prepare the solution freshly on the day of use.

Table 2 — Preparation of standard solutions

Standard solution	Volume taken from stock solution µl	Concentration of aflatoxin ng/ml			
		B1	B2	G1	G2
1	60	15.0	3.75	7.5	3.75
2	40	10.0	2.50	5.0	2.50
3	20	5.00	1.25	2.50	1.25
4	10	2.50	0.625	1.25	0.625
NOTE The values given are for guidance only. The standard range includes the concentrations of the samples.					

5.4. Sulfuric acid, c (H_2SO_4) = 2 mol/l

5.5. Procedure

5.6 Sample preparation and extraction. The sample solution and standard solutions for the HPLC determination shall contain the same solvent or solvent mixture.

Weigh, to the nearest 0.1 g, 25 g of the homogenized test sample into the blender jar (3.2.2). Add 5 g of sodium chloride (4.2) and 125 ml of extraction solvent (4.10) and homogenize with a mixer for 2 min at high speed.

Check that the blending time and speed do not have a negative influence on the extraction efficiency. Filter the mixture through a fluted filter paper (4.2) (V_1).

Pipette 15 ml (V_2) of the filtrate into a conical flask of appropriate size with glass stopper. Add 30 ml of water, stopper the flask and mix. Before starting affinity column chromatography, filter the diluted extract through a glass microfibre filter paper (3.2.4). The filtrate (V_3) should be clear. If not, re-filter it. Proceed immediately in accordance with 5.6.

A centrifuge may also be used to obtain a clear solution.

5.7. Clean-up

Prepare the Immunoaffinity column (3.2.1) and proceed with the clean-up procedure in accordance with the manufacturer's instructions. Pipette 15 ml (V_4) of the second filtrate (V_3) into the solvent reservoir of the Immunoaffinity column. Pass it through the separation column, then wash the column as described in the manufacturer instructions and discard the eluates. Start the elution of the aflatoxins. Collect the methanol or acetonitrile eluate (depending on the product or the manufacturer's instructions) in a 2-ml volumetric flask (3.2.5) (or another volume as specified by the manufacturer). Dilute to the mark with water (V_5). Mix and proceed in accordance with 5.7.

Methods for loading onto Immunoaffinity columns, washing and elution vary slightly between column manufacturers and the specific instructions supplied with the columns should be followed precisely.

NOTE In general, procedures involve sample extraction with a mixture of methanol and water, filtration or centrifugation, possible sample dilution with phosphate buffered solution (PBS) or water, loading under pressure onto a possibly pre-washed column, washing of the column with distilled water and elution of aflatoxins with methanol or acetonitrile (depending on the product and manufacturer's instructions).

Traditional silica gel columns or solid-phase extraction (SPE) columns may also be used. In these cases the manufacturer instructions should also be precisely followed. If the solvent used for elution of aflatoxins is not compatible with the mobile phase, then the eluate should be evaporated into dryness by a Nitrogen stream below 40 °C. The residue should be dissolved in the mobile phase and diluted to 2 ml, or to the volume specified by the manufacturer.

Take care not to exceed the maximum capacity of the column.

GUIDE CHART FOR DETERMINATION OF TOTAL AFLATOXIN CONTENT

SAMPLE PREPARATION

Mix and grind the sample

+5g NaCl

Weigh 25g of homogenised sample

+125ml Extraction Solvent Methanol (HCPL Grade)

Homogenise (2min)

Filtration (Filter paper)

+30ml distilled water

Pipette 15mL Filtrate

Mix (Conical flask)

Filtration (Filter paper with glass microfibres)

Pipette 15 mL Filtrate

+30ml distilled water

Immunoaffinity column

+1mL Methanol

Collect Eluent (1 drop/sec)

+1ml developing solution

Dilute Eluent

HPLC Injection

Condition: Flow rate of mobile phase: 1.0ml/min; Column temperature : 70°C injection volume: 50µl Mobile phase A: Acetonitrile Mobile phase B: Methanol Duration of Analysis: 10-20 minutes Chromatographic column

Calculation of results

5.8. HPLC operating conditions

Connect the separation column outlet to one arm of the T-piece of the post-column derivatization system (4.12) using a short piece of tubing with an internal diameter of, for example, 0.25 mm. Connect the outlet of pump which delivers the post-column derivation reagent to the second arm of the T-piece. Connect one end of a coil of PTFE or stainless steel (see 4.12) to the third arm of the T-piece and connect the other end to the detector (3.2.14). Using an oven or water bath, maintain the reaction coil temperature at 70 °C.

When the column specified in 3.2.12 was used, the following settings were found to be appropriate:
flow rate of mobile phase (column): 1.0 ml/min;
flow rate of post-column reagent: 0.3 ml/min; and
volume injected: 50 µl.

Allow the entire system to run for 10 min to 20 min to stabilize it. If an integrator is used, adjust the sensitivity controls of the fluorescence detector or integrator to give a ratio of 5:1 for signal response: noise for 0.125 ng of aflatoxin G2 in 50 µl. If a strip chart recorder is used, adjust the fluorescence detector control to give 30 % to 40 % scale deflection with 0.125 ng of aflatoxin G2 in 50 µl.

5.9. Identification

Identify each aflatoxin peak in the sample chromatogram by comparing the retention times with those of corresponding reference standards.

Alternatively, the aflatoxins may be identified by simultaneous injection of the sample test solution and standard solutions. Also, the disappearance of the aflatoxin B1 and G1 peaks if no derivatization reagent is added is helpful for identification.

5.10 Calibration graph

Prepare the calibration graph for each aflatoxin by injecting 50 µl of standard solutions 1, 2, 3 and 4 (see Table 2). Check the linearity of the curve for details.

5.11 Determination

Quantitative determination is performed by the external standard method with integration of the peak area or measurement of the peak height, which is then related to the corresponding value for the standard substance. Inject volumes of 50 µl of standard solution into the injection loop, following the instructions of the injector manufacturer. Aflatoxins elute in the order G2, G1, B2, B1 with retention times of approximately 6 min, 8 min, 9 min and 11 min, respectively, and should be baseline-resolved. If necessary, adjust the retention times by changing the methanol concentration of the mobile phase (4.11).

Inject 50 µl (V6) of purified sample extract (5.6) into the injection loop.

5.12 Calculation of results

Calculate the mass; m_t, in grams, of the test sample present in the fraction of the second filtrate taken for the Immunoaffinity column (V_4), using **Equation** (2) below:

$$m_t = m_0 \times \frac{V_2\ V_4}{V_1\ V_3}$$

where

- m_0 is the mass, in grams, of the test portion (5.6), (m_0 = 25 g);
- V_1 is the total volume, in millilitres, of the first filtrate (5.5), (V_1 = 125 ml);
- V_2 is the fraction volume, in millilitres, of the first filtrate (5.5) taken for dilution, (V_2 = 15 ml); V_3 is the total volume, in millilitres, of the second filtrate (5.5), (V_3 = 45 ml);
- V_4 is the fraction volume, in millilitres, of the second filtrate (5.6), (V_4 = 15 ml).

Calculate the mass fraction of each aflatoxin, wi, in micrograms per kilogram of sample, using **Equation (3)** below:

$$W_i = \frac{V_5.m_i}{V_6.m_t}$$

where

- V_5 is the volume, in microlitres, of the eluate (5.6), (V_5 = 2 000 µl);
- V_6 is the volume, in microlitres, of the purified and injected sample extract (5.10), (V_6 = 50 µl);
- mi is the mass, in nanograms, of each aflatoxin i present in the injection volume, corresponding to the measured peak area or peak height read off the calibration graph;
- mt is the mass, in grams, of the test sample present in the fraction of the second filtrate taken for the Immuno
- affinity column (V_4) according to Equation (2).

Add the mass fractions of the four aflatoxins to obtain the mass fraction of total aflatoxins.

6. DETERMINATION OF TOTAL AFLATOXIN USING ELISA

6.1. Principle

ELISA is a solid phase direct competitive enzyme immunoassay. An aflatoxin specific antibody optimized to cross react with all four subtypes of aflatoxin is coated to a polystyrene microwell. Toxins are extracted from a ground sample using a suitable solvent based on the manufacturer's instructions. The extracted sample and conjugated aflatoxins are mixed and added to the antibody-coated microwell. Aflatoxin from the extracted sample and conjugated aflatoxins compete to bind with the antibody coated to the microwell. Microwell contents are decanted and non-specific reactants are removed by washing. An enzyme substrate is added and a distinct colour develops. The intensity of the colour is directly proportional to the amount of bound conjugate and inversely proportional to the concentration of aflatoxin in the sample or standard. Therefore, as the concentration of aflatoxin in the sample or standard increases, the intensity of the colour will decrease. An acidic stop solution is added which changes the chromagen colour. The microwells are measured optically by a microplate reader with an absorbance filter of 450 nm (OD450). The optical densities of the samples are compared to the OD's of the kit standards and an interpretative result is determined.

6.2. Apparatus

- 6.2.1. Antibody coated microwells
- 6.2.2. Mixing wells
- 6.2.3. Aflatoxin controls
- 6.2.4. Conjugate solution
- 6.2.5. Substrate solution
- 6.2.6. Stop solution
- 6.2.7. Grinder sufficient to render sample to particle size of fine instant coffee
- 6.2.8. Collection container, minimum 125-ml capacity
- 6.2.9. Analytical balance
- 6.2.10. Graduated cylinder
- 6.2.11. Analytical solvent in accordance with manufacturer's instructions
- 6.2.12. Distilled or deionized water
- 6.2.13. Filter paper, Whatman #1 or equivalent
- 6.2.14. Filter funnel
- 6.2.15. Pipettor with tips 100 µl and 200 µl
- 6.2.16. Timer
- 6.2.17. Wash bottle (if necessary)
- 6.2.18. Absorbent paper towelse
- 6.2.19. Microplate reader with 450-nm filter

6.3. Precautions

- 6.3.1. Bring all reagents to room temperature (19 oC – 27 oC) before use.
- 6.3.2. Store reagents at conditions recommended by manufacturer.
- 6.3.3.
- 6.3.4.Do not return unused reagents back into their original bottles.
- 6.3.5.
- 6.3.6. Adhere to all time and temperature conditions stated in the procedure.
- 6.3.7.
- 6.3.8. Samples tested should have a pH of 7.0 (±1.0). Excessive alkaline or acidic conditions may affect the test results.
- 6.3.9. Never pipette reagents or samples by mouth.
- 6.3.10. Solvents are flammable.
- 6.3.11. The Stop solution contains acid. Do not allow to contact skin or eyes. If exposed, flush with water.
- 6.3.12. Consider all materials, containers and devices that are exposed to sample or standards to be contaminated with aflatoxin. Wear protective gloves and safety glasses when using this kit.
- 6.3.13. Dispose of all materials, containers and devices in the appropriate receptacle after use.

6.4. Method

Sample preparation, extraction, general procedure and interpretation of results will be determined in accordance with the manufacturer's recommendations.

Produced by EAGC for EAC Partner States

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