

Appendix VII: Determination of Mycotoxins (Aflatoxins)

Mycotoxins, including aflatoxins, refer to the toxic metabolites generated by molds and/or fungi. Since there is concern over the contamination of aflatoxins in CMM, a harmonized method is developed to study the contents of aflatoxins B₁, B₂, G₁ and G₂ in CMM.

Methods –

- (1) **Analysis of aflatoxins** – The analytical procedures must be verified and satisfy with all of the following criteria –
 - (a) the method is suitable for the analysis of the targeted aflatoxins and is not susceptible to the interference from co-extractives;
 - (b) the limits of detection and quantification are determined for each aflatoxin;
 - (c) the limit of quantification for each targeted aflatoxin is set at 0.3 µg/kg;
 - (d) the recovery for each targeted aflatoxin is between 50 and 120%;
 - (e) the repeatability of the method is less than 15% RSD; and
 - (f) a linear response is obtained from the analytical detector within the calibration range.
- (2) **Reagents** – All reagents used should be of analytical grade or equivalent. Methanol and acetonitrile used should be at least of HPLC grade.
- (3) **Apparatus** – All apparatus to be used should be thoroughly cleaned to ensure that they are free from any aflatoxins. Soak the laboratory wares in a solution of household bleach (10%, v/v) for at least 12 h and then wash them with distilled water.
- (4) **Preparation of test sample** – Take a representative CMM sample and cut it into pieces, if necessary, before grinding. Powder the sample before the analysis. Whenever possible, the quantity of the sample to be powdered should be of at least five times as much as those needed for the analysis.
- (5) **Procedure** – The analysis of aflatoxins is based on the detection of the characteristic fluorescence emitted by aflatoxins B₁, B₂, G₁, and G₂ after either post-column iodine and UV excitation or photochemical derivatization. It may have to modify the procedures for the analysis of some samples.

- (a) **Extraction** – Weigh accurately 15.0 g of the blended sample, add 3 g of sodium chloride and 75 mL of a mixture of methanol and water (7:3, v/v). Homogenize the mixture for about 2 min and centrifuge at about $800 \times g$ for 10 min. Check the pH of the extract. Transfer accurately 15 mL of the supernatant solution of the centrifuged mixture to an amber bottle and reduce to about 5 mL with a gentle stream of nitrogen at about 60°C on a water bath [Note 1]. Make up the resultant solution to 50 mL with a solvent that is compatible with the performance of the immunoaffinity column as suggested by the manufacturer [Note 2]. Centrifuge the solution for about 10 min and filter through a glass-fibre filter paper by suction. Collect the filtrate as the test solution.

Note 1: If the sample absorbs solvent significantly (i.e. the volume of the resultant supernatant is less than 40 mL), repeat the extraction as directed above. However, use 5.0 g of sodium chloride and 125 mL of a mixture of methanol and water (7:3, v/v) instead, and transfer 25 mL of the supernatant solution.

Note 2: When handling samples that produce extracts which might affect the normal functioning of the immunoaffinity column, seek manufacturer's advice to ensure the column performance. Pay particular attention to the suggested pH working range of the column and the solvent used for diluting the sample extract prior to column clean-up.

- (b) **Clean-up by immunoaffinity column chromatography** –

Chromatographic procedure – It may be carried out by using –

- an immunoaffinity column containing antibodies specific for aflatoxins B₁, B₂, G₁ and G₂; and
- methanol as the eluting solution.

Performance test of the column – Pass an appropriate volume of the mixed standard solution containing aflatoxins B₁, B₂, G₁ and G₂ through the column, then follow the procedure as described below 'Clean-up of the test solution', and the recovery of the aflatoxins B₁, B₂, G₁ and G₂ should be at least 90%, 80%, 90% and 60% respectively.

Clean-up of the test solution [Note 3] – Condition the column according to the manufacturer's instruction, then pass 22.5 mL of the test solution through the column at a flow rate of about 3 mL/min. Wash the column with 10 mL of eluent, as recommended by the manufacturer, at a flow rate of about 3 mL/min, then wash dry the column by passing 10 mL of air through the column. Elute the column with 1.5 mL of methanol followed by 10 mL of air. Collect all eluates in a 2-mL volumetric flask and make up to the mark with water.

Note 3: It may have to modify the clean-up procedures for different brands of immunoaffinity column. Please refer to manufacturer's instruction.

(c) **Quantitative analysis** – Use a HPLC system that satisfies with all of the following criteria –

- the *R* value of any analyte peak with the adjacent peak: > 1.5;
- the *n* value of any analyte peak: ≥ 7000; and
- the RSD of peak area: ≤ 5%.

Individual aflatoxin standard stock solutions – Determine the concentration of individual aflatoxin stock standard solution (about 10 mg/L) in a mixture of benzene and acetonitrile (98:2, v/v) by UV spectroscopy according to the following equation –

$$\text{Concentration of aflatoxin (mg/L)} = \frac{A_{350} \times M_w \times 1000}{\epsilon}$$

Where A_{350} = the absorbance of the aflatoxin at a wavelength of maximum absorption close to 350 nm,
 M_w = the molecular weight of the aflatoxin (Table 1),
 ϵ = the molar absorptivity of the aflatoxin in benzene-acetonitrile solution (Table 1).

Table 1 The molecular weights (M_w) and molar absorptivities (ϵ) of the aflatoxins

Aflatoxin	Molecular Weight (M_w)	Molar Absorptivity (ϵ)
B ₁	312	19800
B ₂	314	20900
G ₁	328	17100
G ₂	330	18200

Mixed aflatoxins standard solutions – Prepare at least five standard solutions in a mixture of methanol and water (7:3, v/v) containing all the targeted aflatoxins at concentrations suitable for plotting calibration curves.

Chromatographic procedure – It may be carried out by using –

- a LC column (4.6 × 250 mm) packed with particles of octadecylsilyl groups modified silica (5 μm particle size);

- Post-column derivatization
 - (1) Iodine derivatization
 - a post-column reactor system with the reaction temperature set at 70°C and 0.5 mM iodine solution as post-column derivatization reagent. The flow rate of the post-column derivatization reagent is set as 0.3 mL /min;
 - (2) Photochemical derivatization
 - a post-column reactor system with a 254 nm UV lamp and reaction coil for derivatization.
- a mixture of distilled water, acetonitrile and methanol (3:1:1, v/v) as the mobile phase; and
- a fluorescence detector: $\lambda_{\text{ext}} = 360 \text{ nm}$ and $\lambda_{\text{em}} = 450 \text{ nm}$.

Set the flow rate of the mobile phase (LC column) as 1.0 mL/min. Under such conditions, aflatoxins are eluted in the order of G₂, G₁, B₂, and B₁. Calculate the content of each aflatoxin from its peak area and concentration.

Note: Soak all used laboratory wares in a 10% solution of household bleach overnight before reuse or disposal.

Limits – The amount of aflatoxin B₁ and the total amount of aflatoxins (B₁, B₂, G₁ and G₂) in CMM samples should comply with the limits listed in Table 2 below, unless in the case of a CMM of mineral origin or as otherwise specified.

Table 2 The maximum permitted limits of aflatoxins in CMM samples

Aflatoxin	Limit (Not more than)
Aflatoxin B ₁	5 µg/kg
Aflatoxins (sum of B ₁ , B ₂ , G ₁ and G ₂)	10 µg/kg