TOTAL AFLATOXIN ELISA

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A competitive enzyme immunoassay for quantitative analysis of Aflatoxin total in food and feed samples

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12. LITERATURE

1. J.E. Smith, C.W. Lewis and J.G. Anderson. Mycotoxins in human nutrition and health. EU Directorate-General XII, Science, Research and Development, 1990.

2. J.L. Richard, G.A. Bennett, P.F. Ross and P.E. Nelson. Analysis of naturally occurring mycotoxins in feedstuffs and food. J. Anim. Sci. 71, 2563-2574, 1993.

3. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off. J. European Union. L364 (2006) 5-24.

4. Commission Regulation (EU) No 165/2010 of 26 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. Off. J. European Union. L50 (2010) 8-12.

13. ORDERING INFORMATION

For ordering the total Aflatoxin ELISA kit, please use cat. code 5121AFT.

14. LAST MUTATIONS

Kit has been updated. A complete validation was done. The cross reactivities have been validated against new available metabolites.

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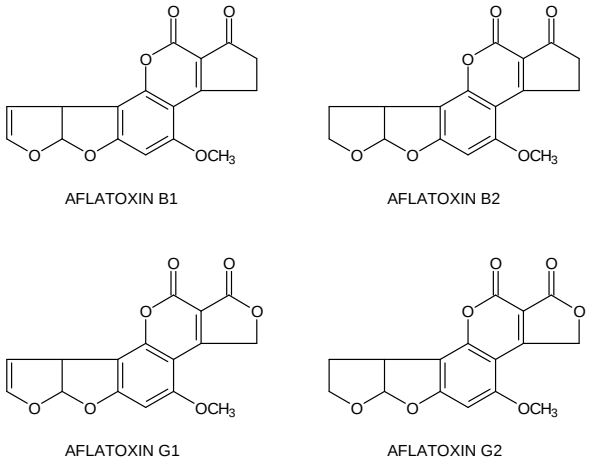
BRIEF INFORMATION

The total Aflatoxin ELISA is a competitive enzyme immunoassay for the screening of food and feed samples. The test is based on antibodies directed against Aflatoxin. The ELISA kit contains a 96 well microtiter plate as well as all essential reagents including ready-to-use standards to perform the test. Methods for a fast and efficient extraction of Aflatoxin from different matrices are included in the kit manual.

1. INTRODUCTION



Chemical structure of Aflatoxins

Aflatoxins (Aflatoxins B_1, B_2, G_1, G_2) are a group of structurally related, extremely toxic compounds produced by the moulds *Aspergillus flavus, A. paraciticus* and *A. nomius* [1]. These moulds occur on food, feed ingredients, derived from tropical and sub-tropical areas. The most pronounced contamination has been found in cereals, rice, maize, soy, tree nuts and peanuts [2].
Within the EU, maximum levels (MLs) for a number of mycotoxins have been set by Commission Regulation (EC) 1881/2006 (which has replaced earlier Regulations (EC) No 1525/98, 194/97 and 466/2001). Concerning the Aflatoxins, this Regulation has been amended by Commission Regulation (EC) No 165/2010. In this amending Regulation, changes in the MLs were needed for alignment with Codex Alimentarius FAO/WHO standards, and new groups of foodstuffs (oilseeds and apricot kernels) are specified. The MLs for Aflatoxin B1 in most of the foodstuffs for direct human consumption vary from 2 to 12 µg/kg (ppb) [3, 4]. However, for processed cereal-based foods, baby foods as well as for dietary foods for special medical purposes the ML for Aflatoxin B1 is set at 0.1 µg/kg (0.1 ppb) [4].

2. PRINCIPLE OF THE TOTAL AFLATOXIN ELISA

The kit is based on a microtiter plate (12 strips, each 8 wells), precoated with rabbit antibodies to mouse IgG. In one incubation step, specific antibodies (mouse anti-Aflatoxin), enzyme labelled Aflatoxin (enzyme conjugate) and Aflatoxin B1 standards or sample are added to the precoated wells. The specific antibodies are bound by the immobilised rabbit antibodies and at the same time free Aflatoxins (in the standard solution or in the sample) and enzyme labelled Aflatoxin compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation of one hour, the non-bound (enzyme labelled) reagents are removed in a washing step. The amount of bound enzyme conjugate is visualised by the addition of chromogen substrate (tetramethylbenzidine, TMB). Bound enzyme transforms the chromogen into a coloured product.
The substrate reaction is stopped by the addition of sulfuric acid. The colour intensity is measured photometrically at 450 nm and is inversely proportional to the Aflatoxin concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

The total Aflatoxin ELISA utilizes antibodies raised in mouse against protein conjugated Aflatoxin. The reactivity pattern of the antibody is:

Cross- reactivity:	Aflatoxin B1	100%
	Aflatoxin B2	112%
	Aflatoxin G1	69%
	Aflatoxin G2	15%
	Aflatoxin M1	16%

The LOD is determined under optimal conditions. Cut-off values need critical consideration.

Matrix	Procedure	LOD ppb
Infant food	8.2	0.016
Brown rice	8.1	0.2
Un-processed cereals	8.1	0.3
Processed cereals	8.1	0.2
Red pepper	8.2	1.0
Liver	8.2	0.05
Serum	8.2	0.025
Feed	8.1	0.4
Nuts	8.1	0.2

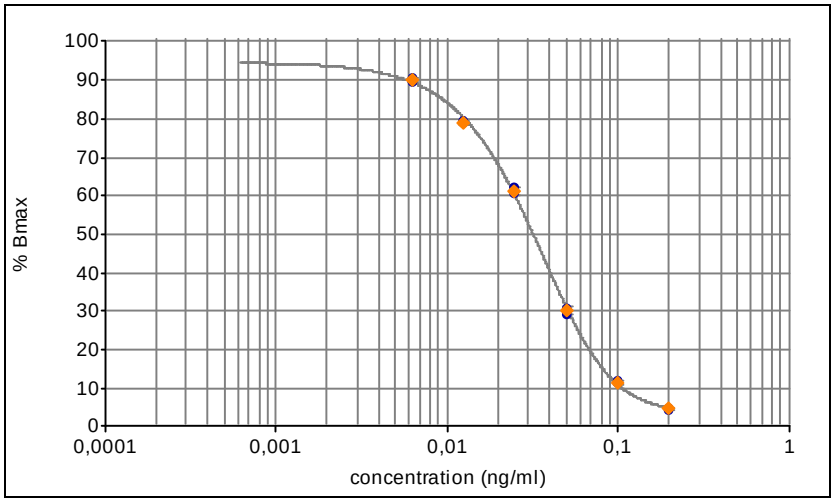


Figure 1 : Example of a calibration curve

The amount of Aflatoxin in the samples is expressed as Aflatoxin B1 equivalents. The Aflatoxin B1 equivalents in the extracts (ng/ml) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

8.1 Food and feed samples
The aflatoxin equivalents, as read from the standard curve, have to be multiplied by a factor 16 to obtain the aflatoxin content in food and feed samples.

8.2 Infant food, cereals and serum
The aflatoxin equivalents, as read from the standard curve, have to be multiplied by a factor 4 to obtain the aflatoxin content in infant food, cereals and serum.

8.2 Liver samples
The aflatoxin equivalents, as read from the standard curve, have to be multiplied by a factor 8 to obtain the aflatoxin content in liver samples.

8.2 Red pepper samples
The aflatoxin equivalents, as read from the standard curve, have to be multiplied by a factor 80 to obtain the aflatoxin content in red pepper samples.

8.3 Procedure to extract fatty samples like, seeds and oil
The concentration of aflatoxin can directly be read from the calibration curve.

If the calculated concentration of aflatoxin exceeds 3 ppb then a further dilution in sample dilution buffer (Chapter 9) is advised.

7. Incubate for 1 hour in the dark at 37°C.

8. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

9. Pipette 100 µl of substrate solution into each well. Incubate 30 minutes at 20°C to 25°C.

10. Add 100 µl of stop solution to each well.

11. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells H1 and H2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the six standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard (Bmax, wells A1 and A2) and multiplied by 100. The zero standard (Bmax) is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

$$\frac{\text{O.D. standard (or sample)}}{\text{O.D. zero standard (Bmax)}} \times 100 = \% \text{ maximal absorbance}$$

Calibration curve:
The values (% maximal absorbance) calculated for the standards are plotted on the Y-axis versus the analyte equivalent concentration (ng/ml) on a logarithmic X-axis.

Alternative for calibration curve
The absorption value of the standards is plotted on the Y-axis versus the concentration on the X-axis. The Y-axis is in logit the Y-axis is logarithmic.

4. HANDLING AND STORAGE

- Kit and kit components are stored in a refrigerator (2°C to 8°C) before and immediately after use.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate at ambient (room) temperature before use.
- Avoid condensation in the wells of the plate. Bring the sealed plate at ambient temperature before opening the plate sealing.
- Any direct action of light on the chromogen solution should be avoided.

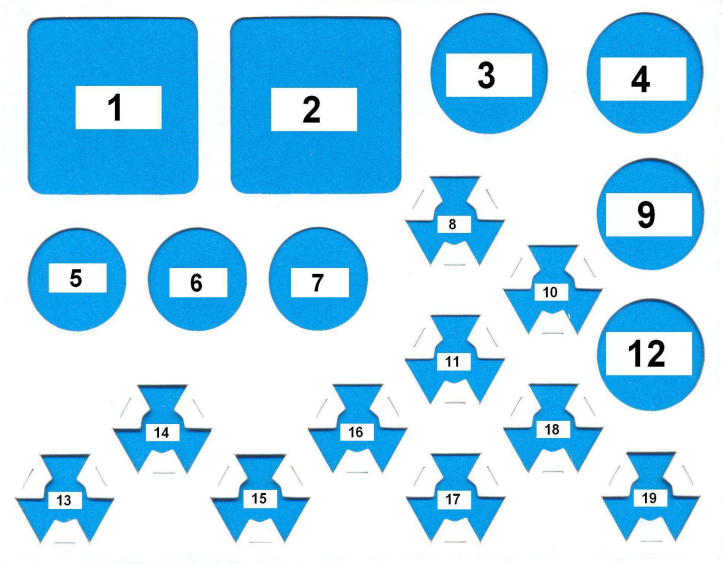
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or absent colour reaction of the maximum binding (zero standard) (E450nm < 0.8).

5. KIT CONTENTS

Manual

1 sealed microtiter plate (12 strips, 8 wells each), coated with antibodies directed against mouse-IgG. Ready-to-use.

Position of the reagents in the kit. For preparation of the reagents see Chapter 9.



1. **Dilution buffer** (20 ml, 4x concentrated)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, Ready-to-use)
4. **Stop solution** (15 ml, Ready-to-use)
5. not in use
6. not in use
7. not in use
8. **Conjugate** (100x concentrated , blue cap)
9. not in use
10. **Antibody** (100x concentrated, yellow cap)
11. not in use
12. not in use
13. **Zero Standard** (2 ml, Ready-to-use)
14. **Standard solution 1** (1ml, Ready-to-use) **0.00625 ng/ml**
15. **Standard solution 2** (1ml, Ready-to-use) **0.0125 ng/ml**
16. **Standard solution 3** (1ml, Ready-to-use) **0.025 ng/ml**
17. **Standard solution 4** (1ml, Ready-to-use) **0.05 ng/ml**
18. **Standard solution 5** (1ml, Ready-to-use) **0.1 ng/ml**
19. **Standard solution 6** (1ml, Ready-to-use) **0.2 ng/ml**

10. ASSAY PROCEDURE

Rinsing protocol

In ELISA's, between each immunological incubation step, unbound components have to be removed efficiently. This is reached by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
Basically, manual rinsing or rinsing with automatic plate wash equipment can be performed as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down and remove residual liquid by striking the plate against a paper towel.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual washing solution in the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment
When using automatic plate wash equipment, check that all wells can be aspirated completely, that the rinsing solution is nicely dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

Assay Protocol

1. Prepare samples according to Chapter 8 (Sample treatment) and prepare reagents according to Chapter 9 (Preparation of reagents).

   Microtiter plate is ready to use.

2. Pipette 100 µl of the zero standard in duplicate (wells H1, H2, blank).
   Pipette 50 µl of the zero standard in duplicate (wells A1, A2; Bmax).
   Pipette 50 µl of each of the Aflatoxin B1 standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.00625, 0.0125, 0.025, 0.05, 0.1 and 0.2 ng/ml).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (40 samples; 80 wells).

4. Add 25 µl of conjugate (Aflatoxin-HRP) to all wells, except wells H1 and H2.

5. Add 25 µl of antibody solution to all wells, except wells H1 and H2.

6. Seal the microtiter plate and shake the plate for a few seconds.

9. PREPARATION OF REAGENTS

Before starting the test, allow the reagents to come to ambient temperature. Any reagents not used should be put back into storage immediately at 2°C to 8°C.

Microtiter plate
Return unused strips into the zip lock bag with desiccant and store at 2°C to 8°C for use in subsequent assays. Retain also the strip holder.

Rinsing buffer
The rinsing buffer is delivered 20 times concentrated. Prepare dilutions freshly before use. For each strip 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 ml distilled water).

Substrate/chromogen solution
The substrate/chromogen solution (ready to use) tends to precipitate at 4°C. Take care that this vial is at room temperature (keep in the dark) and mix the content before pipetting into the wells. Avoid contact with light. Close the vial immediately after use and store at 2°C to 8°C.

*Dilution buffer (4x concentrated)
The dilution buffer is 4 times concentrated. Before dilution (20 ml buffer + 60 ml distilled water) the concentrated buffer should be at room temperature and thoroughly mixed. Concentrated buffer can show precipitates of the contents. Mix well before dilution with distilled water. The 4 times diluted buffer can be stored in a refrigerator (2°C to 8°C) until the expiry date stated on the kit label

**Sample dilution buffer
Prepare freshly before use. Prepare the sample dilution buffer by adding 2 ml of 100% methanol to 8 ml of (4x diluted) dilution buffer.

Conjugate solution (100 µl)
The conjugate is delivered 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 minute, 1000 x g). Add 5 µl of the concentrated conjugate solution to 495 µl (4x diluted) dilution buffer. Per 2 x 8 wells 400 µl is required. Store unused concentrated conjugate at 2°C to 8°C.

Antibody (100 µl)
The antibody is 100x concentrated. Spin down the antibody in the vial by a short centrifugation (1 minute 1000 x g). Add 5 µl of the concentrated antibody to 495 µl (4x diluted) dilution buffer. Per 2 x 8 wells 400 µl of antibody solution is required. Store concentrated antibody immediately upon use at 2°C to 8°C.

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (vortex, mixer)
- Centrifuge (2000 x g)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Test tubes (10 – 15 ml)
- Micropipettes, 25 – 1000 µl
- Multipipette with 2.5 ml combitips
- Aluminium foil or parafilm
- Folded filter
- Methanol
- n-Hexane
- Dichloromethane

7. PRECAUTIONS

- Aflatoxins are carcinogenic compounds. Avoid contact with mouth and skin. Be aware the aflatoxins are not inhaled.
- Any material contaminated with aflatoxins should be destroyed or decontaminated by addition of sodium hypochlorite solution (10% v/v).
- The stop reagent contains 0.5 M sulfuric acid. Do not allow the reagent to get into contact with the skin.
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- TMB is toxic by inhalation, in contact with skin and if swallowed; observe care when handling the substrate.
- Do not use components past expiration date and do not intermix components from different serial lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate, which crystallises at 4ºC.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.

8. SAMPLE TREATMENT

8.1 Feed and Food

- The first step in the sample preparation process depends on the nature of the compound to be tested. In general a homogenous sample has to be obtained from a representative part of the compound. Alternative techniques such as grinding, pulverizing, etc can be used.
- Approximately 50-100 gram of sample is ground and pulverised into a fine homogenous compound
- To 3 gram of ground sample add 9 ml of 80% methanol
- Shake thoroughly at room temperature for 10 minutes
- Centrifuge the sample (10 minutes, 2000 x g, 20°C - 25°C) or filtrate (recommended for larger sample volumes).
- An aliquot of 50 µl of the supernatant obtained after centrifugation, or an aliquot of 50 µl of filtrate obtained after filtration, is diluted with 150 µl of *dilution buffer (4x diluted).
- Pipette 50 µl of the diluted supernatant in the respective wells of the ELISA plate.

8.2 Infant food, red pepper, liver and serum

- The first step in the sample preparation process depends on the nature of the compound to be tested
- In general a homogenous sample has to be obtained from a representative part of the compound. Alternative techniques such as grinding, pulverizing, etc can be used
- Approximately 50 – 100 gram of sample is ground and pulverised into a fine homogenous compound
- Add 10 ml distilled water, to 2 gram of homogenised ground sample and vortex
- Add 20 ml of dichloromethane to the mixture
  Instead of dichloromethane, trichloromethane can be used
- Shake thoroughly, head over head 5 – 10 minutes (Rotor)
- Centrifuge the sample (10 minutes, 2000 x g, 20°C - 25°C)
- Filtrate the layer underneath over a folded filter
- Evaporate 2.5 ml of the filtrate under a mild stream of nitrogen at 50°C
- Dissolve the residue in 1 ml **sample dilution buffer
- Add 1 ml of n-heptane
- Vortex for 1 minute
- Centrifuge (10 minutes, 2000 x g, 20°C - 25°C)
- Pipette 200 µl of the layer underneath into a clean tube
- Dilute with **sample dilution buffer according to the factor per matrix as specified below
- Use an aliquot of 50 µl of the respective dilutions in the ELISA

- For **infant food and cereals**, pipette 50 µl of the layer underneath directly in the respective wells of the ELISA plate. Final dilution is 1 : 4.

- For **liver**, first dilute the layer underneath 1 : 2 in **sample dilution buffer. Then pipette 50 µl of this dilution in the respective wells of the ELISA plate. Final dilution is 1 : 8.

- For **red pepper**, first dilute the layer underneath 1 : 20 in **sample dilution buffer. Then pipette 50 µl of this dilution in the respective wells of the ELISA plate. Final dilution is 1 : 80.

- For **serum,** pipette 50 µl of the layer underneath directly in the respective wells of the ELISA plate. Final dilution is 1 : 4.

Some matrices need a second defatting step. The liquid appears to be turbid after the first defatting step. In that case repeat the defatting procedure.
Repeat the defatting step till the layer underneath is clear. Use 50 µl of the layer underneath in the respective wells of the ELISA plate.

8.3 Procedure to extract fatty samples, like oil, seeds etc.

- Weigh 2 g of finely cut and subsequently homogenised butter or oil into a glass tube.
- Add 8 ml of dichloromethane.
- Mix 30 minutes head over head.
- Filter the butter extract over a folded filter
- Take 4 ml of the filtrate and evaporate to dryness at 50ºC, under a mild stream of nitrogen.
- Dissolve the residue in 1 ml **sample dilution buffer
- Add 1 ml of n-hexane (for defatting) and vortex.
- Centrifuge (5 minutes, 2000 x g, 20°C - 25°C)
- Pipette the layer underneath into a clean glass tube.
- Add 1 ml n-hexane and vortex.
- Centrifuge (5 minutes, 2000 x g, 20°C - 25°C)
- Pipette 50 µl of the layer underneath in the respective wells of the ELISA plate.

*Dilution buffer (kit no.1) 4x concentrated preparation chapter 9.
**Sample dilution buffer. Preparation chapter 9.