ZEARALENONE ELISA

5121ZON[13]06.20

A competitive enzyme immunoassay for
screening on the presence of zearalenone
in various matrices

EUROPROXIMA ZEARALENONE ELISA

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BRIEF INFORMATION

The zearalenone ELISA is a competitive enzyme immunoassay for the screening of cereals and serum samples. The test is based on antibodies directed against zearalenone. 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 zearalenone from different matrices are included in the kit manual.

1. INTRODUCTION

Zearalenone is one of the main mycotoxins which occur in nature. They are produced by a wide variety of *Fusarium* species which may occur on cereals such as wheat, barley, corn, but also on bananas, bean leaves, flax and groundnuts.
Zearalenone is an oestrogenic compound which is also known as F-2 toxin. It causes vulvovaginitis and other oestrogenic responses in swine. In addition to swine, rats, turkey poults and chicks have been shown to be susceptible to the toxic effects of zearalenone.
This Zearalenone assay can be used to detect both zearalenone and its main metabolites α-zearalenol and β-zearalenol. The assay can also detect the anabolic compound zeranol (α-zearalanol) and its main metabolites β-zearalanol (taleranol) and zearalanone. However, these anabolic compounds do not occur in the cereals for which this assay is meant.

2. PRINCIPLE OF THE ZEARALENONE ELISA

The kit is based on a microtiter plate (12 strips, each 8 wells), precoated with sheep antibodies to rabbit IgG. In one incubation step, specific antibodies (rabbit anti-zearalenone), enzyme labelled zearalenone (-HRP) and zearalenone standards or samples are added to the precoated wells. The specific antibodies are bound by the immobilized sheep anti-rabbit antibodies and at the same time free zearalenone (in the standard solution or in the sample) and enzyme labelled zearalenone compete for the specific antibody binding sites (competitive enzyme immunoassay).
After an incubation time of one hour, the non-bound (enzyme labelled) reagents are removed in a washing step. The amount of bound enzyme conjugate is visualized 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 zearalenone concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

Specificity/Cross-reactivity of the antiserum
The antiserum raised in rabbit against zearalenone shows the following cross-reactivities at 50% B/B0:

Zearalenone (F_2-mycotoxin)	100%
α-Zearalenol	75%
β-Zearalenol	30%
Zeranol (α-zearalanol)	150%
Taleranol (β-zearalanol)	60%
Zearalanone	190%
All other steroids tested	<0.01%

The cross-reactivities are determined in a buffer system. The reported values may be different in samples due to matrix effects.
The test cannot discriminate between analytes and cross-reactive substances

The Limit of detection (LOD) is determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	Procedure	LOD ppb
Cereals	8.1	12.5
Milk	8.3	0.625
Milk powder	8.3.1	0.5
Serum	8.4	1.25

If the sample is found to be non-compliant, the results shall be verified by re-analysis of the sample using a confirmatory method.

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- 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 to ambient (room) temperature before use.
- Dilute the kit components immediately before use, but after the components are brought to ambient temperature.
- Avoid condensation in the wells of the plate. Bring the sealed plate to ambient temperature before opening the plate sealing.
- The substrate chromogen solution can be stored in a refrigerator (2ºC to 8ºC) until the expiry date stated on the label.
- Exposure of the chromogen solution to light should be avoided.

Degeneration of the reagents may have occurred when the following phenomena are observed:

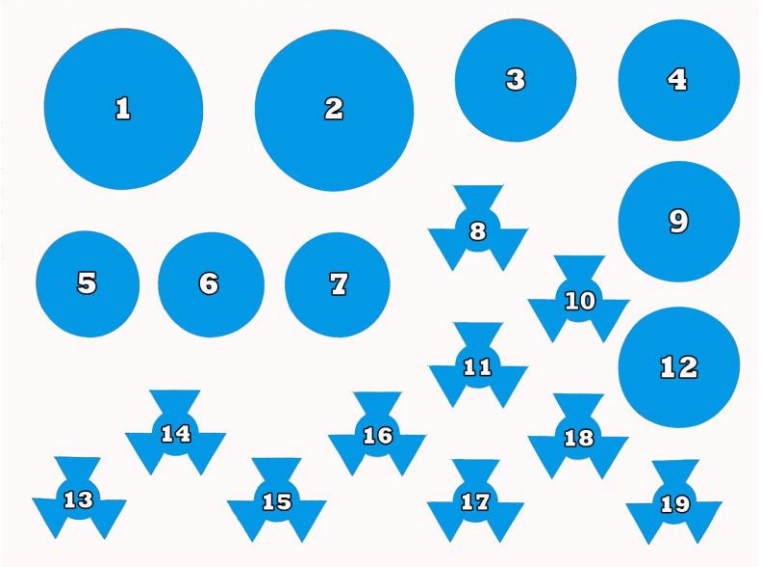
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed microtiter plate (12 strips, 8 wells each), coated with antibodies to rabbit IgG. Plate is 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** (100 µl, 100x concentrated)
9. not in use
10. **Antibody** (100 µl, 100x concentrated)
11. **Standard solution** (100 ng/ml (1 ml, Ready-to-use))
12. not in use
13. **Zero standard solution** (2ml, Ready-to-use)
14. **Standard solution 1** (1ml, Ready-to-use) **0.125 ng/ml**
15. **Standard solution 2** (1ml, Ready-to-use) **0.25 ng/ml**
16. **Standard solution 3** (1ml, Ready-to-use) **0.5 ng/ml**
17. **Standard solution 4** (1ml, Ready-to-use) **1.0 ng/ml**
18. **Standard solution 5** (1ml, Ready-to-use) **5.0 ng/ml**
19. **Standard solution 6** (1ml, Ready-to-use) **10.0 ng/ml**

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
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Acetonitril
- Methanol
- Acetone
- C 18 column (500mg/3 ml)
- Distilled water

7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- 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.
- Do not use components past expiration date and do not use components from different 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 and rinsing buffer, which crystallize 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 PREPARATION

8.1 Cereal samples

Methanol extraction

- Add to 1 g of finely ground sample 4 ml 60% Methanol in distilled water
- Mix 30 minutes, head over head
- Centrifuge the mixture, 10 minutes, 2000 x g
- 25 µl of the aqueous phase is diluted with 475 µl dilution buffer
- 50 µl of this solution is used in the ELISA

8.1.1. Alternative mycotoxin quick test

Acetonitrile extraction

- Add to 5 g of finely ground cereals 20 ml of 84% acetonitrile in distilled water
- Mix 60 minutes, head over head
- The extracts are left to sediment
- 50 µl supernatant is diluted with 450 µl dilution buffer
- 50 µl of this solution is used in the ELISA

8.2. Sensitive test based on liquid extraction and solid phase clean-up

Solid Phase Extraction (SPE)

- Add to 5 g of finely ground cereals 20 ml of 84% acetonitrile in distilled water
- Mix 60 minutes, head over head
- The extracts are left to sediment
- 1 ml of supernatant is evaporated to dryness under a mild stream of nitrogen at 50°C.
- The residue is dissolved in 400 µl methanol 100% and 1600 µl distilled water

Activate the cartridge

- 5 ml methanol 100%
- 5 ml distilled water

Note: It is important that the cartridge is not allowed to dry up completely during activation and prior to sample addition! If the cartridge has become dry, repeat the activation procedure.

- Transfer 2 ml sample onto the activated column (1ml/minute).
- Wash the column
- 3 ml of 20% acetone in water
- 3 ml of 30% methanol in water

Elution of the zearalenone

- Elute sample with 2 x 1 ml of methanol (1ml/minute)
- The methanol is evaporated to dryness under a mild stream of nitrogen at 50°C
- The residue is dissolved in 500 µl of dilution buffer
- 50 µl of this solution is used in the ELISA

8.3. Milk samples
- Pipette 100 µl of homogenized milk sample into a clean tube
- Add 400 µl of dilution buffer, vortex
- 50 µl of this solution is used in the ELISA.

8.3.1 Milk powder samples
- Reconstitute 12 g of the milk powder in 88 ml of distilled water or reconstitute the milk powder according to manufacturers instruction
- Mix well till a homogeneous solution
- Pipette 100 µl of the reconstituted milk sample into a clean tube
- Add 400 µl of dilution buffer, vortex
- 50 µl of this solution used in the ELISA.

8.4 Serum/plasma samples
The samples must be clear, if the samples are turbid, centrifuge (10 minutes, 2000 x g).
- Dilute 50 µl of clear serum/plasma samples with 450 µl dilution buffer, vortex.
- 50 µl of this solution is used in the ELISA.

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 resealable bag with desiccant and store at 2ºC to 8ºC for use in subsequent assays. Retain also the strip holder.

Dilution buffer
The dilution buffer is 4 times concentrated. Before dilution (20 ml + 60 ml distilled water) the concentrated buffer should be at 20°C to 25°C 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 (2ºC to 8ºC) until the expire date stated on the kit label.

Standard 100 ng/ml
To prevent possible matrix effect of the 8.4% acetonitril solution in the sample extract is it advisable to prepare a standard curve in the same matrix.
Prepare first immediately before use 8.4% acetonitril solution in dilution buffer (i.e. 252 µl of 100% acetonitril is added to 2.748 ml dilution buffer).
Dilute standard 100 ng/ml 10x with this 8.4% acetonitril solution (i.e. 50 µl of standard 100 to 450 µl acetonitril solution). Continue to make a dilution range in 8,4% acetonitril solution resulting in a concentration range of 5.0, 2.5, 1.25, 0.25 and 0.125 ng/ml.

Rinsing buffer
The rinsing buffer is delivered 20 times concentrated. Prepare dilutions freshly before use. Per strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Substrate solution
The substrate solution (ready-to-use) precipitates at 4ºC. Take care that this vial is at 20°C to 25°C (keep in the dark) and mix the contents before pipetting into the wells.

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 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 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.

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 rinsing solution from 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 and prepare reagents according to Chapter 9.

2. Pipette 100 µl of zero standard in duplicate (wells H1, H2, blank).

3. Pipette 50 µl of zero standard (Bmax) in duplicate (well A1, A2).

4. Pipette 50 µl of each standard dilution in duplicate (well B1,2 to G1,2, i.e. 0.125, 0.25, 0.5, 1.0, 5.0 and 10.0 ng/ml).

5. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

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

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

8. Seal the microtiter plate and shake the plate for 1 minute.

9. Incubate for 1 hour in the dark at 37ºC.

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

11. Pipette 100 µl of substrate solution into each well.

12. Incubate 30 minutes at 20ºC - 25ºC in the dark.

13. Pipette 100 µl of stop solution to each well.

14. Read the optical density (O.D.) 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.

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

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

Alternative for calibration curve:
The absorbtion 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.

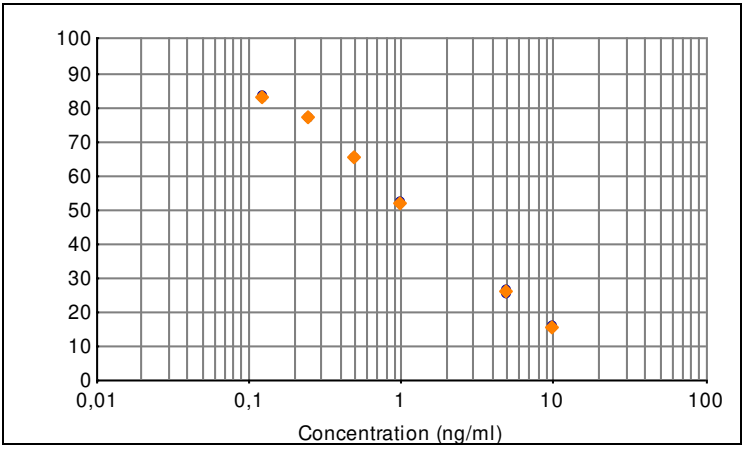


Figure 1 : Example of a calibration curve

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

Mycotoxin quick test

8.1 The calculated zearalenone equivalents have to be multiplied by 100.

8.1.1 The calculated zearalenone equivalents have to be multiplied by 50.

8.2 Solid Phase Extraction (SPE)
The calculated zearalenone equivalents read from the calibration curve have to be multiplied by 2.5.

8.3 Milk samples
The calculated zearalenone equivalents read from the calibration curve have to be multiplied by 5.

8.3.1 Milk powder samples
The calculated zearalenone equivalents read from the calibration curve have to be multiplied by 5.

8.4 Serum/plasma samples
The calculated zearalenone equivalents read from the calibration curve have to be multiplied by 10.

12. LITERATURE

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Bullerman L.B. Significance of mycotoxins to food safety and human health. J. Of Food Protection. 42, 65-86, 1979.
Barna-Vetró I., Gyöngyösi Á. and Solti L. Monoclonal antibody-based enzyme-linked immunosorbent assay of Fusarium T-2 and zearalenone toxins in cereals. Appl. Environ. Microbiol. 60, 729-731, 1994.

13.ORDERING INFORMATION

For ordering the Zearalenone ELISA kit please use cat. code 5121ZON

14. REVISION HISTORY

The manual is adapted to a new layout of the test kit. Several textual changes are added.

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