OCHRATOXIN-A ELISA

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**A competitive enzyme immunoassay
for quantitative analysis of Ochratoxin-A
in food and feed samples**

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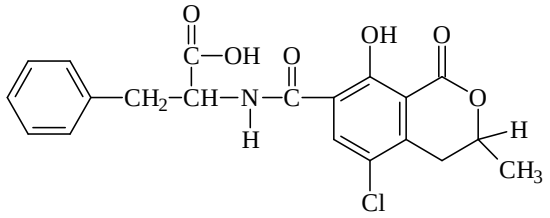
BRIEF INFORMATION

The Ochratoxin-A (OTA) ELISA is a competitive enzyme immunoassay for the screening and quantitative analysis of food and feed samples (corn, rice, wheat, sorghum, barley, oats, rye, coffee, cocoa, dry beans and wine). The test is based on mouse monoclonal antibodies against Ochratoxin-A. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate, which means that in total 40 samples can be analysed. The ELISA kit contains all the reagents, including standards, required to perform the test. Materials and chemicals necessary for the extraction of Ochratoxin from the samples are not included in the test-kit.

1. INTRODUCTION



OCHRATOXIN A

Ochratoxin-A is a nephrotoxic and nephrocarcinogenic mycotoxin produced by *Penicillium verrucosum* and *Penicillium viridicatum* in temporate and cold climates and by a number of *Aspergillus* species such as *A. ochraceus* in warmer and tropical areas of the world. [1]. Ochratoxin-A has been shown to occur in various cereals and other plant products, coffee beans and coffee products, wine, animal feeds. Due to contaminated feed or food the Ochratoxin-A also can occure in pig kidneys, liver and muscle, blood, urine and faeces from pigs and in human blood [2].
For Ochratoxin-A maximum levels (MLs) are established legally in Europe. Depending on the fact whether products are used directly for human consumption or the products still have to be processed. The MLs vary from 3 to 10 µg/kg (ppb) [3,4].

2. PRINCIPLE OF THE OCHRATOXIN-A ELISA

The microtiter plate based ELISA kit consists of 12 strips, each containing 8 wells, precoated with rabbit antibodies to mouse IgG. Specific antibodies (mouse monoclonal anti-Ochratoxin-A), horseradish peroxidase labelled Ochratoxin-A (enzyme conjugate Ochratoxin-A-HRP) as well as Ochratoxin-A standard solutions or samples are added to the precoated wells, followed by a single incubation step.

The specific antibodies are bound by the immobilised rabbit anti-mouse antibodies and simultaneously the Ochratoxin-A-HRP and the Ochratoxin-A present in the standard solutions or in the samples compete for the specific anti-Ochratoxin-A 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 Ochratoxin-A-HRP is visualised by the addition of enzyme substrate/chromogen (peroxide/tetramethylbenzidine, TMB). During the incubation the colourless chromogen is converted by the enzyme into a blue reaction product. This blue colour is inversely proportional to the amount of bound Ochratoxin-A. The more Ochratoxin-A is present in the standard solution or sample, the less colour is developed.
The substrate reaction is stopped by the addition of sulfuric acid. In the acidic environment the blue colour changes into a yellow colour. The colour intensity is measured photometrically at 450 nm.

3. SPECIFICITY AND SENSITIVITY

The Ochratoxin-A ELISA utilizes monoclonal antibodies raised in mouse to protein conjugated Ochratoxin-A. The reactivity pattern of the antibody is:

Cross- reactivities:	Ochratoxin-A	100%
	Ochratoxin-B	9.3%
	Ochratoxin-α	< 0.1%
	Coumarin	< 0.1%
	4-Hydroxy-coumarin	< 0.1%
	D, L-phenylalanine	< 0.1%

The limit of detection (LOD) is calculated as: Xn ± 3SD (n >20)..
The LOD is determined under optimal conditions. Cut-off values need critical consideration.

If a lower LOD is required than a higher amount of sample has to be extracted with dichloromethane.

Matrix	Procedure	LOD ppb
Wine	8.1	0.25
Cereals	8.2	1
Cocoa	8.2	1
Green Coffee	8.2	1

13. ORDERING INFORMATION

For ordering the Ochratoxin-A ELISA kit, please use cat. Code 5121OCH.

14.LAST MUTATIONS

Removed: soluble coffee
Pipette schedule of the standard curve is adapted.

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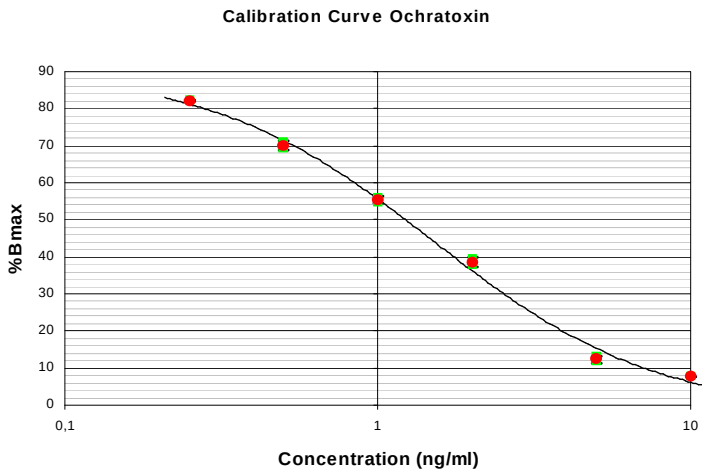


Figure 1 : Example of a calibration curve

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

8.1 Red, rosé and white wine
The ochratoxin equivalents can be read directly from the calibration curve.

8.2 Cocoa, green coffee, cereals
The ochratoxin equivalents can be read directly from the calibration curve and have to be multiplied by a factor 2.5.

12. LITERATURE

1. Kuiper-Goodman. Risk assessment of the mycotoxin Ochratoxin-A Biomedical and Environmental Sciences: 2, 179-248, 1989.
2. Marley E.C., Nicol W.C. and Candlish A.A.G. Determination of Ochratoxin-A by immunoaffinity column clean-up and HPLC in wheat and pig liver. Mycotoxin Research: 11, 111-116, 1995.
3. Commission Regulation (EC) No 466/2001 of 8 March 2001 setting maximum levels for certain contaminants in foodstuffs. Off. J. European Commun. L77 (2001) 1-13.
4. Commission Regulation (EC) No 472/2002 of 12 March 2002 amending Regulation (EC) No 466/2001 setting maximum levels for certain contaminants in foodstuffs.

4. HANDLING AND STORAGE

- Store the kit at +2°C to +8°C in a dark place.

- After the expiration date (see kit label) has passed, it is no longer possible to accept any further quality guarantee.

- Before opening the sealed plate, the plate should be at ambient temperature. This is to avoid condensation in the ELISA plate after the plate is transported from the refrigerator to room temperature.

- Reconstitute or dilute the kit components immediately before use, but after the components are at ambient temperature.

- After the lyophilised kit components have been reconstituted, these components have to be used directly or can be stored in a refrigerator for maximally one week (stored at + 2°C to + 8°C in the dark). Alternatively, after reconstitution of the antibody and conjugate components, aliquots of these solutions can be prepared. The aliquots can be stored in a freezer (–20°C) for at least one year.

- The substrate and standard solutions can be stored in a refrigerator (+2°C to +8°C) until the expiration date stated on the label.

- Any direct action of light on the substrate/chromogen solution should be avoided.

If the following phenomena are observed, this may indicate a degradation of the reagents.

- A blue colouring of the substrate/chromogen solution before putting it into the wells.

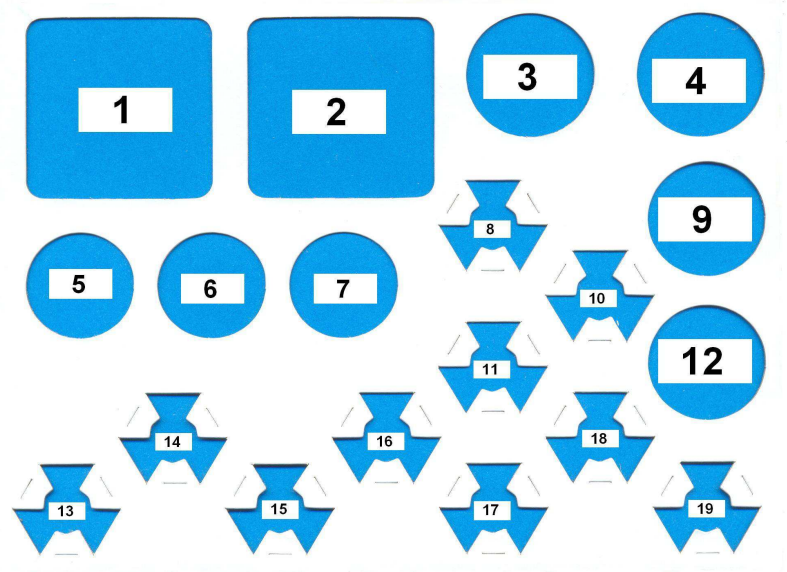
- A weak or absent colour reaction of the maximum binding (zero standard) (E450nm < 0.8).

5. KIT CONTENTS

Manual

One sealed microtiter plate (12 strips, 8 wells each), coated with antibodies to mouse 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, 20 times concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (15 ml, ready-to-use)
5. **Conjugate** (lyophilised, blue cap)
6. **Antibody** (lyophilised, yellow cap)
7. **Standard** (lyophilised, black cap)
8. not in use
9. **Extraction buffer A** (13 ml, 5x concentrated, white cap)
10. not in use
11. not in use
12. not in use
13. not in use
14. not in use
15. not in use
16. not in use
17. not in use
18. not in use
19. not in use

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 (Ochratoxin-A-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.
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 min. at room temperature (20ºC - 25ºC).
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 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 (wells A1 and A2) and multiplied by 100. The zero standard is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

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

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.

Conjugate solution
Reconstitute the vial of lyophilised conjugate (Ochratoxin-A-HRP) with 4 ml of dilution buffer, mix thoroughly and keep in the dark until use.
Store the vial immediately after use in the dark at +2°C to +8°C.

Antibody solution
Reconstitute the vial of lyophilised antibodies with 4 ml of dilution buffer, mix thoroughly and keep in the dark until use.
Store the vial immediately after use in the dark 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 done 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).

2. Pipette 100 µl of dilution buffer in duplicate (well H1, H2).
   Pipette 50 µl of dilution buffer in duplicate (well A1, A2).
   Pipette 50 µl of each standard dilution in duplicate (well B1,2 to G1,2).

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (blender, Ultra Turrax, mixer)
- Centrifuge (for 10 - 30 ml test tubes, with cooling, 2500 x g)
- Vortex
- Horizontal shaker
- Automated microplate washer or 8-channel micropipette 100 – 300 µl
- Magnetic stirrer
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Erlenmeyer flasks 50 ml
- Glass test tubes (10 – 30 ml)
- Micropipettes 20 – 200 µl, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Aluminium foil or parafilm
- Dichloromethane (CH_2Cl_2)
- Polypropylene tubes Greiner 210 261
- Diethylether $(CH_3CH_2)_2O$
- Rotor
- Nitrogen
- n-Hexane $CH_3(CH_2)_4CH_3$

7. PRECAUTIONS

- Ochratoxin-A is carcinogenic and toxic compound. Avoid contact with mouth and skin. Be aware the ochratoxins are not inhaled.
- Any material contaminated with Ochratoxin-A should be destroyed or decontaminated.
- The stop reagent contains 0.5 M sulphuric 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 precision and accuracy.

8. SAMPLE TREATMENT

8.1 Extraction procedure for red, rosé and white wine

- Pipette 1 ml of wine in a tube (polypropylene Greiner 210 261)
- Add 5 ml dichloromethane (CH_2Cl_2)
- Mix head over head 5-10 minutes (Rotor)
- Allow the mixture to separate in two layers for 10 minutes
- Remove the upper layer
- Take 1 ml of the layer underneath and evaporate to dryness under a mild stream of nitrogen at 50ºC
- Dissolve the residue in 0.2 ml dilution buffer (supplied in the kit)
- Pipette 50 µl into the wells of the ELISA plate

8.2 Extraction procedure for cocoa, green coffee, cereals

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 gram of sample is ground and pulverised into a fine homogenous compound.
- Weigh 5 gram homogenized sample into a clean tube (polypropylene Greiner 210 261)
- Add 10 ml phosphoric acid, 0.5 M
- Mix head over head 10 minutes (Rotor)
- Add 20 ml dichloromethane (CH_2CL_2)
- Mix head over head 10 minutes (Rotor)
- Centrifuge the mixture 5 minutes, 2000 x g
- Remover the upper layer (phosphoric acid)
- Vortex the mixture
- Centrifuge the mixture 5 minutes, 2000 x g
- Filtrate the layer underneath (dichloromethane phase) trough a paper filter
- Pipette 12 ml of the filtrate into a glass tube and evaporate to dryness under a mild stream of nitrogen at 50ºC

- Dissolve the residue in 1.5 ml extraction buffer (A)
- Add 2 ml n-Hexane $CH_3(CH_2)_4CH_3$
- Mix head over head for 1 minute
- Centrifuge 5 minutes 2000 x g
- Remover the upper layer (n-Hexane)
- Diluted 50 µl of the layer underneath with 200 µl dilution buffer (delivered with the kit)
- Pipette 50 µl of the diluted lower layer into the wells of the ELISA plate

9. PREPARATION OF REAGENTS

The reagents included in the test-kit are sufficient to carry out at least 96 analyses (including standard analyses). Each standard and sample is analysed in duplicate

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

Prepare reagents freshly before use

Microtiter plate
Return unused strip into the resealable 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).

Extraction buffer (5x concentrated)
The extraction buffer is 5 times concentrated. Diluted 13 ml extraction buffer + 52 ml distilled water. The 5 times diluted buffer can be stored in a refrigerator (+2°C to +8°C).

Dilution buffer (4x concentrated)
The sample 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.

Substrate solution
The substrate solution (ready to use) precipitates 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.

Standard solutions
Prepare a dilution range of the Ochratoxin-A standard.
Add 2.0 ml of dilution buffer to the vial of the Ochratoxin-A standard and mix. This Ochratoxin-A solution contains 10 ng/ml.
Continue to make a dilution range of 5, 2, 1, 0.5 and 0.25 ng/ml in dilution buffer.
Store the vial immediately after use in the dark at +2°C to +8°C.