OCHRATOXIN A ELISA

(5121OTA[1]03.17)

A competitive enzyme immunoassay
for quantitative analysis of
Ochratoxin A in various matrices

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13. ORDERING INFORMATION

For ordering the OTA ELISA kit, please use cat. code 5121OTA.

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Beijerinckweg 18
NL 6827 BN Arnhem
The Netherlands

TEL: + 31 26 3630364
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E-mail: info@europroxima.com

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells G1 and G2 (blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the five 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 (Bmax) is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

O.D. standard (or sample)
---------- x 100 = % maximal absorbance
O.D. zero standard

Calibration curve:
The values (% maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the ochratoxin A equivalent concentration (ng/ml) on a logarithmic X-axis. The calibration curve should be virtually linear in the 0.0625 – 0.5 ng/ml range.

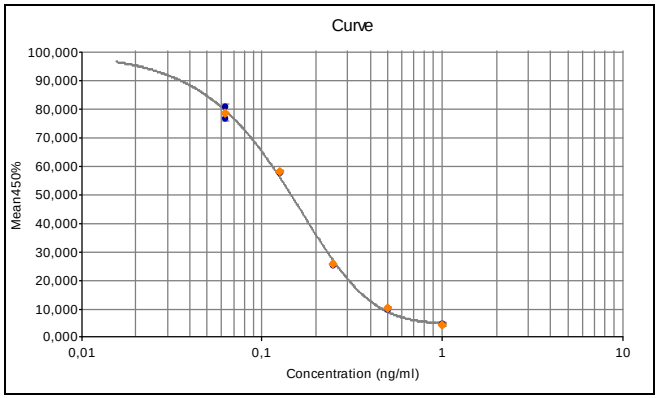


Figure 1 : Example of a calibration curve

The amount of ochratoxin A in the extracted sample is expressed as ochratoxin A equivalents. The ochratoxin A equivalents (ng/ml) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

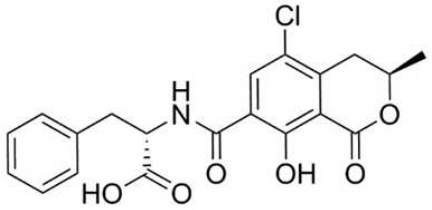
Calculation factors

Cereals (procedure 8.1), roasted and instant coffee (procedure 8.3), green coffee (procedure 8.4) and cocoa (procedure 8.5)
To calculate the concentration of ochratoxin A in cereals, coffee and cocoa expressed in ppb (ng/g) the ochratoxin A equivalents read from the calibration curve should be multiplied by a factor 25.

BRIEF INFORMATION

The Ochratoxin A (OTA) ELISA is a competitive enzyme immunoassay for the screening and quantitative analysis of different commodities. The test is based on mouse monoclonal antibodies against ochratoxin A. Samples and standards are measured in duplicate. A total of 41 samples can be analysed with one kit. The ELISA kit contains all the reagents, including standards, required to perform the test. Materials and chemicals necessary for the extraction of ochratoxin A 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 temperate 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, and feed. In the European Union maximum levels (MLs) for ochratoxin A have been set for different food commodities. The MLs vary from 0.5 to 20 µg/kg (ppb) depending on the food type [2]. The guidance limits for feed are 250 µg/kg for cereals and cereal products and 50 and 100 µg/kg for complete and complementary feedingstuffs for pigs and poultry, respectively [3].

Maximum limits for ochratoxin A in food in the EU:

Commodity	ML [ppb]	Commodity	ML [ppb]
Unprocessed cereals	5	Wine and grape juice	2
Processed cereals	3	Baby food and dietary food	0.5
Dried vine fruit	10	Pepper, nutmeg, ginger, turmeric	15
Roasted coffee	5	Chili, cayenne, paprika	20
Instant coffee	10	Liquorice	20

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-HRPO) 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 60 minutes, 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/tetramethyl benzidine, 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 sulphuric acid. In the acidic environment the blue colour changes into yellow. 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 cross-reactivity pattern of the antibody is:

Ochratoxin A	100%
Ochratoxin B	18%

The limit of detection (LOD) is calculated as: $mean_{blank}$ + 3×SD (n =20).
The LOD is determined under optimal conditions. Cut-off values need critical consideration.

Matrix	Procedure	LOD [ppb]
Wheat	8.1	1.7
Corn	8.1	1.4
Wine and must	8.2	0.3
Roasted coffee	8.3	1.9
Instant coffee	8.3	1.8
Green coffee	8.4	1.2
Cocoa	8.5	1.7

10. ASSAY PROCEDURE

Rinsing protocol
Unbound components have to be removed efficiently between each incubation step in ELISAs. This is achieved by appropriate rinsing. Each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
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 followed by a firm short vertical movement.
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 the zero standard in duplicate (wells G1, G2, blank).
   Pipette 50 µl of the zero standard in duplicate (wells A1, A2; Bmax).
   Pipette 50 µl of each of the ochratoxin A standard solutions in duplicate (wells B1,2 to F1,2 i.e. 0.0625, 0.125, 0.25, 0.5 and 1 ng/ml).
3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (41 samples; 82 wells).
4. Add 25 µl of conjugate (Ochratoxin A-HRP) to all wells, except wells G1 and G2.
5. Add 25 µl of antibody solution to all wells, except wells G1 and G2.
6. Seal the microtiter plate and shake the plate for a few seconds.
7. Incubate for 60 minutes in the dark at room temperature (20ºC - 25ºC).
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 room temperature (20ºC - 25ºC).
10. Add 100 µl of stop solution to each well.
11. Read the absorbance values immediately at 450 nm.

KH_2PO_4 0.18 g
NaCl 8.94 g
pH 7.4 (7.2 – 7.5)

**Methanol:PBS buffer (50:50)
This buffer is used for the extraction of cereals (procedure 8.1). Mix equal volumes of methanol and PBS buffer, for example 50 ml of methanol with 50 ml of PBS buffer.

***Carbonate/bicarbonate buffer (carb/bicarb buffer), 50 mM
This buffer is used during extraction of wine (procedure 8.2), coffee (procedures 8.3 and 8.4) and cocoa (procedure 8.5).
500 ml of water
Na_2CO_3 0.795 g
$NaHCO_3$ 1.465 g
pH 9.6-9.8

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place.
- 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.
- Exposure of the chromogen solution to light should be avoided.

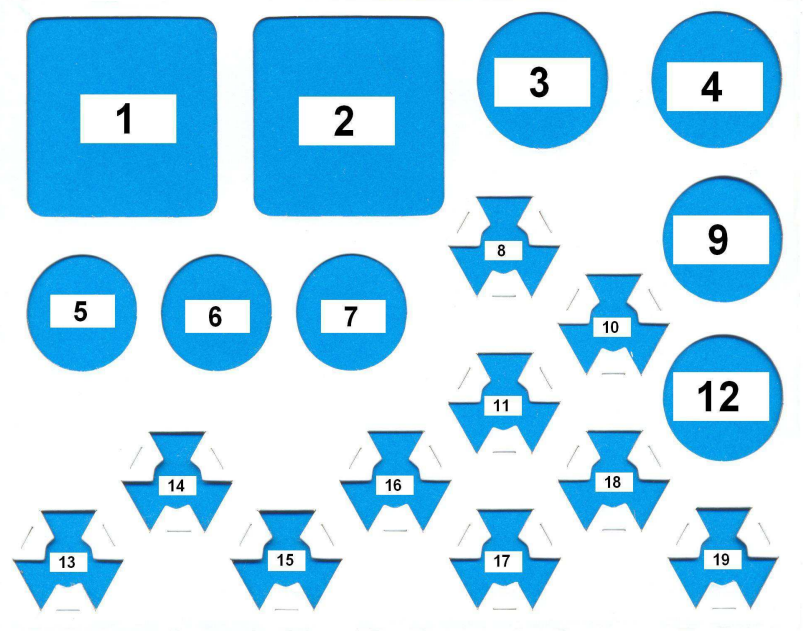
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

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

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



1. **Dilution buffer** (40 ml)
2. **Rinsing buffer** (30 ml, 20× 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× concentrated, blue cap)
9. not in use
10. **Antibody** (100× concentrated, yellow cap)
11. not in use
12. not in use
13. **Zero standard** (2 ml, Ready-to-use)
14. **Standard solution 1** (1 ml, Ready-to-use 0.0625 ng/ml)
15. **Standard solution 2** (1 ml, Ready-to-use 0.125 ng/ml)

9. PREPARATION OF REAGENTS

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

Before starting the assay, reagents should be brought up to ambient temperature (20°C - 30°C). Any reagents not used should be put back into storage immediately at 2°C to 8°C.

Prepare reagents freshly before use

Microtiter plate
Bring the plate to ambient temperature before opening to avoid condensation in the wells. Return unused strips into the resalable 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 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).

Dilution buffer
Dilution buffer is ready-to-use.

Substrate solution
The substrate solution (ready-to-use) tends to precipitate at 4°C. Make sure that this vial is at room temperature before use (keep in the dark) and mix the content before pipetting into the wells.

Conjugate solution (100 µl)
Prepare reagents freshly before use. The conjugate (Ochratoxin A-HRP) is 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 min, 1000 x g). Add 5 µl of the concentrated conjugate solution to 495 µl of dilution buffer (chapter 5, no.1). Per 2 x 8 wells 400 µl of diluted conjugate is required. Store unused concentrated conjugate at 2°C to 8°C.

Antibody solution (100 µl)
Prepare reagents freshly before use. The antibody is 100x concentrated. Spin down the antibody in the vial by a short centrifugation step (1 minute, 1000 x g). Add 5 µl of the concentrated antibody solution to 495 µl of dilution buffer (chapter 5, no.1). Per 2x 8 wells 400 µl of diluted antibody is required. Store unused concentrated antibody at 2°C to 8°C.

*Phosphate buffered saline (PBS)
PBS buffer is needed to prepare methanol:PBS buffer (50:50) for the extraction of cereals (procedure 8.1).
1 l of water
Na_2HPO_4 0.77 g

8.4. Extraction procedure for green coffee

- Weigh 2 g of homogenized coffee into a 50 ml polypropylene tube
- Add 10 ml of 0.1 M phosphoric acid and 10 ml of dichloromethane
- Vortex for 5 sec and then mix head-over-head for 15 min
- Centrifuge for 2 min at 4000 × g
- Collect the bottom layer and filter it through a filter paper
- Pipette 2 ml of the filtrate into a 15 mL polypropylene tube and add 1 ml of carb/bicarb buffer***
- Vortex for 5 sec and mix head-over-head for 5 min
- Centrifuge for 5 min at 4000 × g
- Collect 50 µl of the upper layer and add to 450 µl of the dilution buffer (supplied in the kit), vortex
- Centrifuge for 5 min at 4000 × g
- Pipette 50 µl of the clear supernatant into the wells of the ELISA plate

8.5. Extraction procedure for cocoa powder

- Weigh 2 g of cocoa powder into a 50 ml polypropylene tube
- Add 10 ml of dichloromethane and 200 µl of 6 M phosphoric acid
- Vortex for 5 sec and then mix head-over-head for 15 min
- Centrifuge for 2 min at 4000 × g
- Filter the supernatant through a filter paper
- Pipette 2 ml of the filtrate into a 15 ml polypropylene tube and add 1 ml of carb/bicarb buffer***
- Vortex for 5 sec and mix head-over-head for 5 min
- Centrifuge for 5 min at 4000 × g
- Collect 50 µl of the upper layer and add to 450 µl of the dilution buffer (supplied in the kit), vortex
- Centrifuge for 5 min at 4000 × g
- Pipette 50 µl of the clear supernatant into the wells of the ELISA plate

*PBS buffer, pH 7.4, see chapter 9
**Methanol:PBS buffer (50:50), see chapter 9
***Carbonate/bicarbonate buffer, 50 mM, pH 9.6-9.8, see chapter 9

16. **Standard solution 3** (1 ml, Ready-to-use 0.25 ng/ml)
17. **Standard solution 4** (1 ml, Ready-to-use 0.5 ng/ml)
18. **Standard solution 5** (1 ml, Ready-to-use 1 ng/ml)
19. not in use

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Vortex
- Head-over-head shaker
- Centrifuge
- Automated microplate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes 20 – 200 µl, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Aluminium foil or parafilm
- Dichloromethane (CH_2Cl_2)
- Methanol (anhydrous)
- n-Hexane
- Phosphoric acid 6 M
- Phosphoric acid 0.1 M
- Carbonate/bicarbonate buffer 50 mM, pH 9.6-9.8
- PBS buffer, pH 7.4
- Polypropylene tubes, 15 mL
- Polypropylene tubes, 50 mL
- Eppendorf tubes, 2 mL

7. PRECAUTIONS

- Ochratoxin A is carcinogenic and toxic compound. Avoid contact with mouth and skin. Avoid breathing dust.
- 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 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; be careful when handling the substrate.
- Do not use the components if it is past their expiration date and do not mix components from different lot numbers.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, protect from damage and dirt.
- All components should be completely dissolved before use. Pay 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 cereals (wheat and corn)

- Weigh 2 g of homogenized cereal into a 50 ml polypropylene tube
- Add 8 ml of methanol:PBS buffer** (50:50) and 2 ml of n-hexane
- Vortex for 5 sec and then mix head-over-head for 15 min
- Centrifuge for 5 min at 4000 × g
- Remove the upper (hexane) layer
- Collect 100 µl of the lower layer and add to 400 µl of the dilution buffer (supplied in the kit), vortex
- Centrifuge for 5 min at 4000 × g
- Pipette 50 µl of the clear supernatant into the wells of the ELISA plate

8.2 Extraction procedure for wine (red and white) and must

- Pipette 1 ml of wine or 1 g of must into a 15 ml polypropylene tube
- Add 2 ml of dichloromethane (CH_2Cl_2) and 20 µl of 6 M phosphoric acid
- Vortex for 5 sec and then mix head-over-head for 15 min
- Allow the mixture to separate in two layers for 5 min
- Remove the upper layer
- Pipette 1 ml of the lower dichloromethane layer into a new tube (2 mL Eppendorf tube). In case of must there might be no upper layer formed, in this happens just collect 1 ml of clear dichloromethane. Add 0.5 ml of carb/bicarb buffer***
- Vortex for 5 sec and mix head-over head for 5 min
- Centrifuge for 2 min at 4000 × g
- Collect 100 µl of the upper layer and add to 300 µl of the dilution buffer (supplied in the kit), vortex
- Pipette 50 µl of the sample into the respective wells of the ELISA plate

8.3. Extraction procedure for ground roasted and instant coffee

- Weigh 2 g of coffee into a 50 ml polypropylene tube
- Add 10 ml of dichloromethane and 50 µl of 6 M phosphoric acid
- Vortex for 5 sec and then mix head-over-head for 15 min
- Filter through a filter paper
- Pipette 2 ml of the filtrate into a 15 ml polypropylene tube and add 1 ml of carb/bicarb buffer***
- Vortex for 5 sec and mix head-over-head for 5 min
- Centrifuge for 5 min at 4000 × g
- Collect 50 µl of the upper layer and add to 450 µl of the dilution buffer (supplied in the kit), vortex
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Subtract the mean optical density (O.D.) of the wells G1 and G2 (blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the five 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 (Bmax) is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

O.D. standard (or sample)
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Calibration curve:
The values (% maximal absorbance) calculated for the standards are plotted (on the Y-axis) versus the ochratoxin A equivalent concentration (ng/ml) on a logarithmic X-axis. The calibration curve should be virtually linear in the 0.0625 – 0.5 ng/ml range.

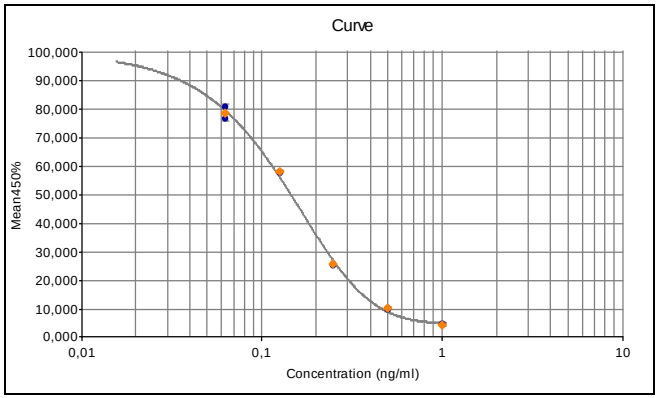

Figure 1 : Example of a calibration curve

The amount of ochratoxin A in the extracted sample is expressed as ochratoxin A equivalents. The ochratoxin A equivalents (ng/ml) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

Calculation factors

Cereals (procedure 8.1), roasted and instant coffee (procedure 8.3), green coffee (procedure 8.4) and cocoa (procedure 8.5)
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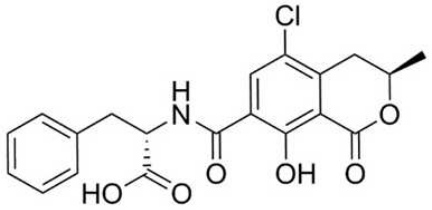

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Maximum limits for ochratoxin A in food in the EU:

Commodity	ML [ppb]	Commodity	ML [ppb]
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Dried vine fruit	10	Pepper, nutmeg, ginger, turmeric	15
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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 followed by a firm short vertical movement.
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 the zero standard in duplicate (wells G1, G2, blank).
   Pipette 50 µl of the zero standard in duplicate (wells A1, A2; Bmax).
   Pipette 50 µl of each of the ochratoxin A standard solutions in duplicate (wells B1,2 to F1,2 i.e. 0.0625, 0.125, 0.25, 0.5 and 1 ng/ml).
3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (41 samples; 82 wells).
4. Add 25 µl of conjugate (Ochratoxin A-HRP) to all wells, except wells G1 and G2.
5. Add 25 µl of antibody solution to all wells, except wells G1 and G2.
6. Seal the microtiter plate and shake the plate for a few seconds.
7. Incubate for 60 minutes in the dark at room temperature (20ºC - 25ºC).
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 room temperature (20ºC - 25ºC).
10. Add 100 µl of stop solution to each well.
11. Read the absorbance values immediately at 450 nm.

KH_2PO_4 0.18 g
NaCl 8.94 g
pH 7.4 (7.2 – 7.5)

**Methanol:PBS buffer (50:50)
This buffer is used for the extraction of cereals (procedure 8.1). Mix equal volumes of methanol and PBS buffer, for example 50 ml of methanol with 50 ml of PBS buffer.

***Carbonate/bicarbonate buffer (carb/bicarb buffer), 50 mM
This buffer is used during extraction of wine (procedure 8.2), coffee (procedures 8.3 and 8.4) and cocoa (procedure 8.5).
500 ml of water
Na_2CO_3 0.795 g
$NaHCO_3$ 1.465 g
pH 9.6-9.8

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place.
- 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.
- Exposure of the chromogen solution to light should be avoided.

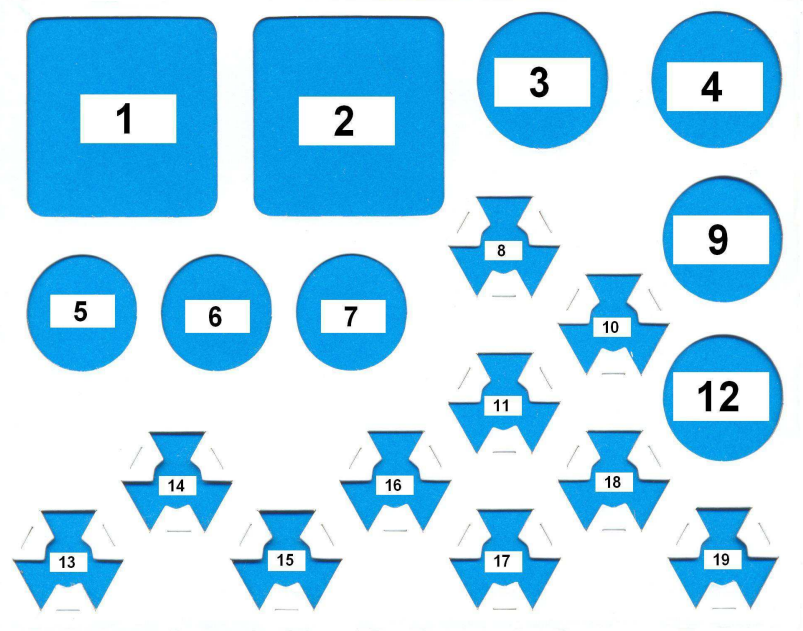
Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual

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

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



1. **Dilution buffer** (40 ml)
2. **Rinsing buffer** (30 ml, 20× 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× concentrated, blue cap)
9. not in use
10. **Antibody** (100× concentrated, yellow cap)
11. not in use
12. not in use
13. **Zero standard** (2 ml, Ready-to-use)
14. **Standard solution 1** (1 ml, Ready-to-use 0.0625 ng/ml)
15. **Standard solution 2** (1 ml, Ready-to-use 0.125 ng/ml)

9. PREPARATION OF REAGENTS

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

Before starting the assay, reagents should be brought up to ambient temperature (20°C - 30°C). Any reagents not used should be put back into storage immediately at 2°C to 8°C.

Prepare reagents freshly before use

Microtiter plate
Bring the plate to ambient temperature before opening to avoid condensation in the wells. Return unused strips into the resalable 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 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).

Dilution buffer
Dilution buffer is ready-to-use.

Substrate solution
The substrate solution (ready-to-use) tends to precipitate at 4°C. Make sure that this vial is at room temperature before use (keep in the dark) and mix the content before pipetting into the wells.

Conjugate solution (100 µl)
Prepare reagents freshly before use. The conjugate (Ochratoxin A-HRP) is 100x concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 min, 1000 x g). Add 5 µl of the concentrated conjugate solution to 495 µl of dilution buffer (chapter 5, no.1). Per 2 x 8 wells 400 µl of diluted conjugate is required. Store unused concentrated conjugate at 2°C to 8°C.

Antibody solution (100 µl)
Prepare reagents freshly before use. The antibody is 100x concentrated. Spin down the antibody in the vial by a short centrifugation step (1 minute, 1000 x g). Add 5 µl of the concentrated antibody solution to 495 µl of dilution buffer (chapter 5, no.1). Per 2x 8 wells 400 µl of diluted antibody is required. Store unused concentrated antibody at 2°C to 8°C.

*Phosphate buffered saline (PBS)
PBS buffer is needed to prepare methanol:PBS buffer (50:50) for the extraction of cereals (procedure 8.1).
1 l of water
Na_2HPO_4 0.77 g

8.4. Extraction procedure for green coffee

- Weigh 2 g of homogenized coffee into a 50 ml polypropylene tube
- Add 10 ml of 0.1 M phosphoric acid and 10 ml of dichloromethane
- Vortex for 5 sec and then mix head-over-head for 15 min
- Centrifuge for 2 min at 4000 × g
- Collect the bottom layer and filter it through a filter paper
- Pipette 2 ml of the filtrate into a 15 mL polypropylene tube and add 1 ml of carb/bicarb buffer***
- Vortex for 5 sec and mix head-over-head for 5 min
- Centrifuge for 5 min at 4000 × g
- Collect 50 µl of the upper layer and add to 450 µl of the dilution buffer (supplied in the kit), vortex
- Centrifuge for 5 min at 4000 × g
- Pipette 50 µl of the clear supernatant into the wells of the ELISA plate

8.5. Extraction procedure for cocoa powder

- Weigh 2 g of cocoa powder into a 50 ml polypropylene tube
- Add 10 ml of dichloromethane and 200 µl of 6 M phosphoric acid
- Vortex for 5 sec and then mix head-over-head for 15 min
- Centrifuge for 2 min at 4000 × g
- Filter the supernatant through a filter paper
- Pipette 2 ml of the filtrate into a 15 ml polypropylene tube and add 1 ml of carb/bicarb buffer***
- Vortex for 5 sec and mix head-over-head for 5 min
- Centrifuge for 5 min at 4000 × g
- Collect 50 µl of the upper layer and add to 450 µl of the dilution buffer (supplied in the kit), vortex
- Centrifuge for 5 min at 4000 × g
- Pipette 50 µl of the clear supernatant into the wells of the ELISA plate

*PBS buffer, pH 7.4, see chapter 9
**Methanol:PBS buffer (50:50), see chapter 9
***Carbonate/bicarbonate buffer, 50 mM, pH 9.6-9.8, see chapter 9

16. **Standard solution 3** (1 ml, Ready-to-use 0.25 ng/ml)
17. **Standard solution 4** (1 ml, Ready-to-use 0.5 ng/ml)
18. **Standard solution 5** (1 ml, Ready-to-use 1 ng/ml)
19. not in use

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Vortex
- Head-over-head shaker
- Centrifuge
- Automated microplate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes 20 – 200 µl, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Aluminium foil or parafilm
- Dichloromethane (CH_2Cl_2)
- Methanol (anhydrous)
- n-Hexane
- Phosphoric acid 6 M
- Phosphoric acid 0.1 M
- Carbonate/bicarbonate buffer 50 mM, pH 9.6-9.8
- PBS buffer, pH 7.4
- Polypropylene tubes, 15 mL
- Polypropylene tubes, 50 mL
- Eppendorf tubes, 2 mL

7. PRECAUTIONS

- Ochratoxin A is carcinogenic and toxic compound. Avoid contact with mouth and skin. Avoid breathing dust.
- 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 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; be careful when handling the substrate.
- Do not use the components if it is past their expiration date and do not mix components from different lot numbers.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, protect from damage and dirt.
- All components should be completely dissolved before use. Pay 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 cereals (wheat and corn)

- Weigh 2 g of homogenized cereal into a 50 ml polypropylene tube
- Add 8 ml of methanol:PBS buffer** (50:50) and 2 ml of n-hexane
- Vortex for 5 sec and then mix head-over-head for 15 min
- Centrifuge for 5 min at 4000 × g
- Remove the upper (hexane) layer
- Collect 100 µl of the lower layer and add to 400 µl of the dilution buffer (supplied in the kit), vortex
- Centrifuge for 5 min at 4000 × g
- Pipette 50 µl of the clear supernatant into the wells of the ELISA plate

8.2 Extraction procedure for wine (red and white) and must

- Pipette 1 ml of wine or 1 g of must into a 15 ml polypropylene tube
- Add 2 ml of dichloromethane (CH_2Cl_2) and 20 µl of 6 M phosphoric acid
- Vortex for 5 sec and then mix head-over-head for 15 min
- Allow the mixture to separate in two layers for 5 min
- Remove the upper layer
- Pipette 1 ml of the lower dichloromethane layer into a new tube (2 mL Eppendorf tube). In case of must there might be no upper layer formed, in this happens just collect 1 ml of clear dichloromethane. Add 0.5 ml of carb/bicarb buffer***
- Vortex for 5 sec and mix head-over head for 5 min
- Centrifuge for 2 min at 4000 × g
- Collect 100 µl of the upper layer and add to 300 µl of the dilution buffer (supplied in the kit), vortex
- Pipette 50 µl of the sample into the respective wells of the ELISA plate

8.3. Extraction procedure for ground roasted and instant coffee

- Weigh 2 g of coffee into a 50 ml polypropylene tube
- Add 10 ml of dichloromethane and 50 µl of 6 M phosphoric acid
- Vortex for 5 sec and then mix head-over-head for 15 min
- Filter through a filter paper
- Pipette 2 ml of the filtrate into a 15 ml polypropylene tube and add 1 ml of carb/bicarb buffer***
- Vortex for 5 sec and mix head-over-head for 5 min
- Centrifuge for 5 min at 4000 × g
- Collect 50 µl of the upper layer and add to 450 µl of the dilution buffer (supplied in the kit), vortex
- Centrifuge for 5 min at 4000 × g
- Pipette 50 µl of the clear supernatant into the wells of the ELISA plate