Enzyme immunoassay for the detection of ochratoxin A

(code OR360/OR361)

***I'screen* OCHRA** is a kit prepared for an immunoenzymatic assay for the quantitative analysis of ochratoxin.
The kit contains the procedure and the materials sufficient for 96 determinations ***(code OR360)*** or 48 determinations ***(code OR361)*** including standards.
A microtiter plate photometer or a strip photometer is required.

Analysable samples
Cereals/feed, wine, grapes, cocoa, green coffe.

Sample preparation
Cereals/feed (acqueous extraction): Grinding, extraction with sodium bicarbonate sol., filtration or centrifugation
Cereals/feed (solvent extraction) cocoa, green coffe, wine, grapes,: (grinding of cereals, green coffe and cocoa; blending of grapes), acidification, extraction with solvent, extraction with sodium bicarbonate solution, dilution (if indicated).

Assay time: 40 minutes (sample preparation not included).

Detection limit
Cereals/feed, cocoa: 1 ppb
Wine, grapes. 0,1 ppb
Green coffe: 0,2 ppb.

Specificity

Molecule	cross-reactivity
Ochratoxin A	100 %
Ochratoxin B	1,65 %
Zearalenone	<0,1 %
Aflatoxin B1	<0,1 %

1 TEST PRINCIPLE

The assay is performed in polystyrene microwells which have been coated with antibodies (IgG) anti-IgG of rabbit. Ochratoxin A standard solutions or samples, the enzyme conjugate ochratoxin-HRP and a specific antibody anti-ochratoxin are added to the microwells. During the incubation, free ochratoxin-A molecules and ochratoxin-HRP compete for the anti-ochratoxin antibodies binding sites.
The anti-ochratoxin antibodies are simultaneously bound to the solid fase. Any unbound enzyme ochratoxin-HRP is then removed in a washing step. The bound enzyme (HRP) activity is determined by adding a fixed amount of a chromogenic substrate. The enzyme converts the colourless chromogen into a blue product. The addition of the stop reagent leads to a color change from blue to yellow. The absorbance is measured by a microplate reader at 450 nm. The colour development is inversely proportional to the ochratoxin-A concentration in the sample.

2 MATERIALS PROVIDED

Microtiter plate: coated with antibodies IgG anti-IgG of rabbit.
Code OR360: 96 wells (12 strips X 8 wells)
Code OR361: 48 wells (6 strips X 8 wells)
As the strips are breakable, the wells can be used individually. For this purpose, it is sufficient to get out the well from the sheath and to break the joint.
Std Ochratoxin A: 7 amber glass vials containing 1,5 ml of an ochratoxin A solution in the following concentrations: 0 ng/ml; 0,05 ng/ml; 0,1ng/ml; 0,3 ng/ml; 1 ng/ml; 2,5 ng/ml; 5 ng/ml. White cap.
Enzyme Conjugate: 1 amber glass vial containing 250 ul.
Enzyme Conjugate diluent: 1 glass bottle. Red cap. Code OR360: containing 20 ml; code OR361: containing 10 ml.
Anti-ochratoxin A antibody: 1 glass bottle containing 9 ml. Blue cap.
Washing-buffer 10 x: 1 plastic bottle containing 50 ml.
Developing solution: 1 amber plastic bottle containing 24 ml.
Stop solution; 1 glass bottle containing 9 ml. White cap.

3 REQUIRED BUT NOT PROVIDED MATERIALS

- Distilled or deionized water.
- Sodium bicarbonate solution 0,13 M, pH 8,1 (fill up 1,09 g NaHCO3 with distilled water to 100 ml).
- Chloroform; 0,1M phosphoric acid; celite (Sigma Code C-8656) for green coffe and cocoa samples.
- 1M HCl, dichlormethane (for wine, grape, cereals and feed samples)

Equipment
- Grinder (for cereals,feed, green coffe and cocoa)
- Blender or shaker (for all matrices)
- Centrifuge (for all matrices)

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- Filter paper (for all matrices)
- Balance (for cereals,feed, green coffe and cocoa)
- Fume hood
- 20-200 µl micropipettes with tips.
- 50-300 µl multichannel micropipette with tips.
- Adsorbent paper
- Microtiter plate or strip reader equipped with a 450 nm filter.

4 PRECAUTIONS AND ADVICES

- For *in vitro* diagnostic use only.
- Some reagents contain preservative. The stop solution contains sulphuric acid and is corrosive. Standard solutions contain trace amount of ochratoxin. Due to its concentration (values are printed on the label), these solutions are classified as not hazardous.
- Handle the reagents with caution, avoiding contact with skin, eyes and mucous membranes.
- Safety data sheet available on Tecna's web site.

5 STORAGE INSTRUCTIONS

- Store the kit at +2/+8 °C and do not freeze components.
- Reseal the unused strips of the microtiter plate in the bag together with the dessicant bag provided.
- Do not use components after the expiration date.
- Do not use fotocopies of the instruction booklet. Keep always to the instruction booklet which is included inside the kit.
- Do not intermix components between different kit lots.
- Prepare samples using preferably glass materials.

6 SAMPLES PREPARATION

6.1 Green coffe

- Finely grind the sample.
- To 5 g of powder add 10 ml of 0,1M phosphoric acid.
- Add 100 ml of chloroform and 2 g of celite.
- Shake on a low speed shaker (400 rpm) for 15 minutes; let stand.
- Filter on 01 Whatman filter paper.
- To 50 ml of filtrate add 10 ml of the sodium bicarbonate solution. Shake for 30 seconds, then let the separation of the two phases. Take the upper acqueous phase and centrifuge it to remove solvent traces. Use the surnatant in the test. The dilution factor is 4.

6.2 Cereals and feed

6.2.1. Acqueous Extraction

- Weight 5 g of the finely grinded sample.
- Add 100 ml of sodium bicarbonate solution prepared according to point 3.
- Shake with Ultraturrax or blender for 1 minute at 12000 rpm or for 15 minutes with a low speed shaker (400 rpm)
- Centrifuge at 1700xg (at room temperature) for 15 minutes or filter (Whatman 1). Use the surnatant in the test. The dilution factor is 20.

6.2.2. Organic solvent extraction

- Add 15 ml of HCl 1 M to 5 g of finely grinded sample.
- Add 30 ml dichloromethane.
- Shake for 15 minutes on a low speed shaker (400 rpm).
- Let separate the liquid from the solid phase, then transfer 5 ml of the lower dichlormethane phase in a tube.
- Add 5 ml of the sodium bicarbonate solution (point 3) and shake for 15 minutes on a low speed shaker (400 rpm).
- Centrifuge for 15 minutes at 2200xg.
- Take 150 µl the upper acqueous phase and dilute with 350 µl of the bicarbonate solution. The dilution factor is 20.
- Note: if the acqueous phase is not clear (emulsion), centrifuge again, preferably with a Labnet or Eppendorf microcentrifuge on 12000 rpm. Take 150 µl the upper clear acqueous phase and dilute with 350 µl of the bicarbonate solution. The dilution factor is 20.

6.3 Red wine, white wine, grapes

- Put the grapestones into a blender and mix throughly.
- Equilibrate the wine at room temperature, and if necessary, degas it under vacuum for 15 minutes.
- Add 5 ml of HCl 1 M to 5 ml of the wine sample (or to 5 g of the grapes sample). From this point the preparation procedure is equal for the two matrices.
- Add 10 ml dichloromethane.
- Shake for 15 minutes on a low speed shaker (400 rpm).
- Centrifuge for 15 minutes at 2200xg. Two phases are formed: a lower solvent and an upper acqueous.
- Take 5 ml of the solvent phase and add 2,5 ml of the sodium bicarbonate solution (point 3) and shake for 15 minutes on a low speed shaker (400 rpm).
- Centrifuge for 15 minutes at 2200xg.
- Take the upper acqueous phase and to separate it from any residual solvent centrifuge again for 15 minutes at 2200xg.
- Take the acqueous phase and dilute it 2x with the bicarbonate solution. (for example: 1 ml of acqueous phase + 1 ml of sodium bicarbonate). The dilution factor is 2.

6.4. Cocoa

- Finely grind the sample.
- To 2,5 g of powder add 5 ml of 0,1M phosphoric acid, 50 ml of chloroform and 2,5 g of celite.
- Shake on a low speed shaker (400 rpm) for 15 minutes; let stand.
- Filter on 01 Whatman filter paper.
- Collect 25 ml of filtrate and add 5 ml of the sodium bicarbonate solution. Shake for 30 seconds, then let the separation of the two phases. Take the upper acqueous phase and centrifuge it to remove solvent traces. To 1 ml of surnatant add 4 ml of the sodium bicarbonate solution. The dilution factor is 20.

7 WORKING SOLUTIONS PREPARATION

Ochratoxin-A standard solutions: ready to use.
Enzyme Conjugate diluent: ready to use.
Enzyme conjugate: ATTENTION: in order to recover the total coniugate amount, before use, centrifuge the vial briefly at low speed (spin-dow).
Calculate and prepare the quantity necessary for the experiment. Dilute the conjugate **1/200** with the enzyme diluent (for example, 20 µl of enzyme conjugate concentrate + 3980 µl of enzyme conjugate diluent).
Mix gently by inverting the vial. DO NOT VORTEX.

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Do not take the Enzyme Conjugate concentrate in volume lower of 20 µl.

Antibody anti-ochratoxin A: ready to use.

Washing buffer: dilute the concentrate 1:10 (1+9) with distilled water. ATTENTION: in presence of crystals, bring the solution at room temperature and stir in order to solve them completely.

The diluted washing buffer is stable at room temperature for 24 hours and at +2/+8°C for two weeks.

Developing solution: ready to use; this solution is light sensitive: keep away from direct light.

Stop solution: ready to use.

8 ASSAY PROCEDURE

8.1 Preliminary comments

- Before use, let stand all reagents at room temperature for approximately 1 hour.
- Return all reagents to +2/+8 °C immediately after use.
- Do not change the assay procedure, in particular:
  - do not prolong the first incubation time.
  - **incubate the plate at room temperature (18 - 25°C).**
  - do not shake the plate during the incubations.
  - use for dispensing accurate and precise micropipettes with suitable tips.
- Once started, complete all the steps without interruption.
- The reproducibility of ELISA results depends largely upon the efficiency and uniformity of microwells washing; always keep to the described procedure;
- Use a single disposable tip for each standard and sample to avoid cross-contamination.
- Do not allow tips to contact the liquid already present in the microwells or the internal microwells surface.
- Avoid direct sunlight during all incubations. Cover the microtiter plate without the sealing tape.

8.2 Assay procedure

1. Predispose an assay layout, recording the standard and samples positions, taking into account that all have to be run in duplicate;
2. Add 50 ul of each standard/sample into the standard/sample wells
3. Using the multichannel micropipette, add 100 ul of enzyme conjugate in each well;
4. Using the multichannel micropipette, add 50 ul of antibody in each well and shake the plate gently with rotatory motion for few seconds.
5. Incubate 20 minutes at room temperature.

Do not prolong the first incubation time.

6. Washing sequence
   - Pour the liquid out from the wells.
   - Using a squeeze bottle, fill completely all the wells with working wash solution using a squeeze bottle. Pour the liquid out from the wells.
   - repeat the washing sequence four (4) times.

     Remove the remaining droplets by tapping the microplate upside down vigorously against absorbent paper;

Do not allow the wells to dry out.

7. Using the multichannel micropipette, add 200 ul of developing solution to the well and mix thoroughly with rotatory motion for few seconds.
8. Incubate for 20 minutes at room temperature.
9. Using the multichannel micropipette, add 50 ul of stop solution to each well and mix thoroughly with rotatory motion for few seconds.
10. Measure the absorbance at 450 nm. Read within 60 minutes.

In case a strip reader is used, it is necessary to take out the strip from the frame and to remove the case round the wells.

9 RESULTS CALCULATION

- Calculate the mean absorbance of the each standards and samples;
- Divide the mean absorbance value of each standard and sample by the mean absorbance of the Standard 0 (B_O) and multiply by 100; the Maximum Binding is thus made equal to 100% and the absorbance values are quoted in percentage:

$$\frac{\text{standard (or sample) absorbance}}{\text{absorbance Standard 0 } (B_O)} \times 100 = \frac{B}{Bo}\ (\%)$$

- Enter the (%) B/B_O values calculated for each standard against the ochratoxin A standards concentration in a semi-logarithmic system of coordinates; draw the standard curve;
- Interpolate the B/B_O value for each sample to the corresponding concentration 0n the calibration curve.
- The concentration of ochratoxin A present in the samples is obtained from the concentration read from the calibration curve multiplied by the dilution factor. Following the procedures of sample preparation mentioned (chapter 8), the dilution factor for green coffe is 4 for cereals/feed is 20, for wine/grape is 2, for cocoa is 20.

Please note: For calculation of the ELISA results, Excel spreadsheets can be downloaded from the section "download" in Tecna's web site, www.tecnalab.com.

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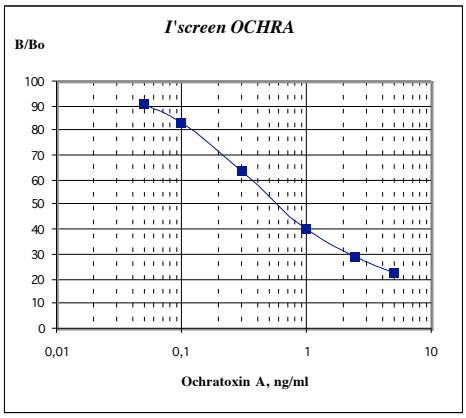
10 EXAMPLE OF STANDARD CURVE



11 RESULTS EVALUATION

After results elaboration, it is necessary to verify the assay performance. The verification is performed by comparision of obtained data with those given in kit specifications. If the values are out from the specifications given, it is advised to control the expiry date of the kit, the wavelenght of absorbance recordering, as well as the procedure employed. If operation errors do not emerge, contact our technical assistance. WARNING: substitution will be possible just in case of rendered kit. The kit must be conserved in its integral version and at the temperature indicated in this booklet.

12 KIT SPECIFICATIONS

12.1 Assay specification

Mean Bo absorbance	$\geq$ 0,7 OD_{450nm}
B/Bo 50 %	0,2 - 2 ng/ml
Std duplicates mean C.V. (%)	$\leq$ 6 %

12.2 Assay performance

Matrix	LOQ ppb	CUT OFF ppb
Green coffe	0,5	$\leq$ 0,2
Wheat, barley (procedure 6.2.1.)	2	1,5
Wine and grapes	0,2	$\leq$ 0,1
Cocoa	1	$\leq$ 1

13 BIBLIOGRAPH

Angelini E., Bazzo I., Savino M., Borgo M. (2008). Ochratoxin A: Comparison of Extraction Methods from Grapes and Quantitative Determination by Different Competitive Enzyme-Linked Immunosorbent Assay Kits. Journal of Food Protection, 71(12): 2488-2496.

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Persic,L., Perrone,P. and Paleologo Oriundi,M. Rapid and sensitive screening of ochratoxin A in wine samples by competitive ELISA. Poster presentation at the VIIth International Conference on Agri-Food Antibodies. Sept.10-13, 2003. Uppsala, Sweden.

14 LIABILITY

Samples evaluated as positive using the kit have to be re-tested with a confirmation method.

Tecna shall not be liable for any damages to the customer caused by the improper use of the kit and for any action undertaken as a consequence of results.

Tecna shall not be liable for the unsafe use of the kit out of the current European safety regulations.

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