

Importance of Total Ochratoxin Determination

Ochratoxins are known contaminants of human food and animal feed produced by certain ubiquitously occurring *Aspergillus* and *Penicillium* species. The toxins are frequently detected in cereals, coffee, beer, wine, pet food, etc. Ochratoxins have been shown to be nephrotoxic, teratogenic and carcinogenic in laboratory animals.

Ochratoxin A (OTA) is the most prominent toxin, followed by ochratoxin B (OTB) and ochratoxin C (OTC). OTB has been reported to be less toxic than OTA, however, more recent data brings this into question. OTA and OTB can occur simultaneously, as they are produced by the same species of fungus and to date several studies have demonstrated synergistic effects of these toxins *in vitro* and *in vivo*. Based on those findings, it can be assumed that OTB may pose a risk to humans. Therefore, OTB is as least as important as OTA and to minimize risk it might be concluded that routine detection of OTB and OTA in food and beverages would be a prerequisite for improved determination of ochratoxin risk.

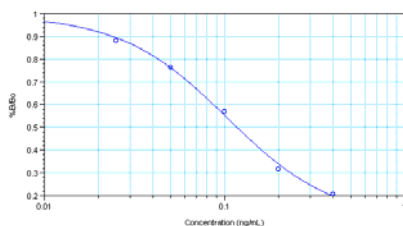
To protect consumers from mycotoxin related risks, the European Union (EU) has established regulatory limits for OTA, (10 ppb in dried vine fruits and instant coffee, 5 ppb in cereals and roasted coffee, 2 ppb in wine. No regulatory limits exist currently for OTB or OTC.

The Abraxis Total Ochratoxins ELISA allows the determination of 42 samples in duplicate determination. Only a few milliliters of sample are required. The test can be performed in 90 minutes.

Performance Data

Test sensitivity:

The limit of detection for Ochratoxin A in buffer calculated as 90% B/Bo is equal to <0.03 ng/mL. The concentration of residue necessary to cause 50% inhibition (50% B/Bo) is approximately 0.12 ng/mL. Determinations closer to the middle of the calibration range of the test yield the most accurate results. The following is the sensitivity in different matrices: 2.5 ppb in green/roasted coffee; 1.25 ppb in grains; 0.5 ppb in beer; 1.75 ppb in wine; 5.0 ppb in dried pet food.



Test reproducibility: Coefficients of variation (CVs) for standards: <10%; CVs for samples: <15%.

Selectivity: This ELISA recognizes Ochratoxin A and related compounds with varying degrees:

Cross-reactivities:	Ochratoxin A	100%
	Ochratoxin B	231%
	Ochratoxin C	118%
	Fumonisin-B1	<0.2%
	Fumonisin-B2	<0.2%
	Deoxynivalenol	<0.2%
	Zearalonone	<0.2%

Samples: To eliminate matrix effects in samples, a sample extraction/clean-up and dilution is required. See Preparation of Samples section. For additional extraction procedures for various other matrices please contact Abraxis LLC.

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Ochratoxins ELISA, Microtiter Plate

Enzyme-Linked Immunosorbent Assay for the Determination of Total Ochratoxins (A/B/C) in Contaminated Samples

Product No. 53020BA

1. General Description

The Ochratoxins ELISA is an immunoassay for the detection of total Ochratoxins (A/B/C). This test is suitable for the quantitative and/or qualitative detection of Ochratoxins in contaminated samples. Positive samples should be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions in this test kit contain small amounts of Ochratoxin A in solution. In addition, the substrate solution contains tetramethylbenzidine and the stop solution contains diluted sulfuric acid. Avoid contact of standard and stopping solutions with skin and mucous membranes. If these reagents come in contact with the skin, wash with water.

3. Storage and Stability

The Ochratoxins ELISA Kit should be stored in the refrigerator (4–8°C) prior to use. The solutions must be allowed to reach room temperature (20–25°C) before use. Reagents may be used until the expiration date on the box. Some reagents need to be stored frozen after reconstitution (Test Preparation, section C).

4. Test Principle

The test is a direct competitive ELISA based on the recognition of Ochratoxins by specific antibodies. Ochratoxins, when present in a sample and a Ochratoxin-enzyme conjugate compete for the binding sites of anti-Ochratoxins antibodies which are immobilized on the wells of the microtiter plate. After a washing step and addition of the substrate solution, a color signal is produced. The intensity of the blue color is inversely proportional to the concentration of Ochratoxins present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the Ochratoxins ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in samples, test interferences caused by matrix effects can not be completely excluded. Mistakes in handling the test can also cause errors. Possible sources for such errors can be:

Inadequate storage conditions of the test kit (or reagents), incorrect pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, extreme temperatures during the test performance (lower than 10°C or higher than 30°C).

The Abraxis Ochratoxins ELISA kit provides screening results. As with any analytical technique (GC, HPLC, etc.), positive samples requiring some action should be confirmed by an alternative method.

Working Instructions

A. Materials Provided

1. Microtiter plate coated anti-Ochratoxins antibody, in a resealable foil pouch with desiccant.
2. Ochratoxin Standards (6): 0, 0.025, 0.05, 0.10, 0.20, and 0.40 ng/mL.
3. HRP Conjugate Assay Buffer, 6 mL.
4. Dilution Buffer I, 25 mL. Used to dilute samples.
5. Ochratoxin-HRP Conjugate, 6 mL.
6. Dilution Buffer II, 25 mL.
7. Wash Solution (5X) Concentrate, 100 mL.
8. Color (Substrate) Solution (TMB), 16 mL.
9. Stop Solution, 12 mL.

B. Additional Materials (not included with the test kit)

1. Micro-pipettes with disposable plastic tips (10-200 and 200-1000 μL)
2. Multi-channel pipette (50-250 μL) or stepper pipette with plastic tips (10-250 μL)
3. Disposable pipettes, 2.0, 5.0, 10 mL
4. Microtiter plate reader (wave length 450 nm)
5. Timer
6. Tape or Parafilm
7. 15 mL and 2.0 mL plastic centrifuge tube or equivalent
8. Distilled or deionized water
9. Methanol
10. Sodium Bicarbonate, ACS grade
11. Vortex mixer and overhead mixer
12. Centrifuge capable of 3,000 x g (13,000 rpm optional)
13. Analytical balance, 2 decimal place

C. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette or a stepping pipette for adding the assay buffer, conjugate, substrate and stop solutions in order to equalize the incubations periods of the solutions on the entire microtiter plate. Please use only the reagents and standards from one package lot in one test, as they have been adjusted in combination.

1. Adjust the microtiter plate and the reagents to room temperature before use.
2. Remove the number of microtiter plate strips required from the foil bag. The remaining strips should be stored in the foil bag and zip-locked closed. Store the remaining kit in the refrigerator (4-8°C).
3. The standards, conjugate assay buffer, conjugate, substrate, Dilution Buffer I and II, and stop solutions are ready to use and do not require any further dilutions.
4. Prepare 5.0% sodium bicarbonate stock solution: 5.0 gm of NaHCO_3 in 100 mL dH₂O. Use the 5% stock solution to dilute to 0.1% with a 1:1 solution of Methanol(MeOH)/DI water.
5. Dilute the wash buffer (5X) concentrate at a ratio of 1:5. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water.
6. The stop solution should be handled with care as it contains diluted H₂SO₄.

D. Preparation of Samples

Samples should be analyzed immediately after preparation to prevent adsorption/degradation of the analyte. Please inquire about preparation of samples for other matrices such as baby food, baby cereals, etc.

Roasted/Green Coffee

1. Weigh 0.5 gm of ground/homogenized coffee into a 15 mL plastic centrifuge tube.
2. Add 10.0 mL of a 1:1 MeOH/DI-0.1% bicarbonate sol., vortex thoroughly. Mix using an overhead rotator for 10 minutes.
3. Centrifuge for 5 min at 3,000 X g. Save supernatant.
4. Dilute supernatant 1:5 (i.e. 200 μL of supernatant, 700 μL dH₂O, and 100 μL of Dilution Buffer I). Vortex to mix and analyze as sample (Assay Procedure, step 1). The Ochratoxins concentration contained in samples is then determined by multiplying the ELISA result by the dilution factor of 100. Recoveries were 75-114%.

Grains

1. Weigh 0.5 gm of homogenized grains into a 15 mL plastic centrifuge tube.
2. Add 5.0 mL of a 1:1 MeOH/DI-0.1% bicarbonate sol., vortex thoroughly. Mix using an overhead rotator for 10 minutes.
3. Centrifuge for 5 min at 3,000 X g. Save supernatant.
4. Dilute supernatant 1:5 (i.e. 200 μL of supernatant, 700 μL dH₂O, and 100 μL of Dilution Buffer I). Vortex to mix and analyze as sample (Assay Procedure, step 1). The Ochratoxins concentration contained in grain samples is then determined by multiplying the ELISA result by the dilution factor of 50. Recoveries were 81-96%.

Beer

1. Measure and add 1.0 mL of beer into a 15 mL plastic centrifuge tube.
2. Add 4.0 mL of a 1:1 MeOH/DI-0.1% bicarbonate, vortex thoroughly. Mix using an overhead rotator for 10 minutes.
3. Centrifuge for 5 min at 3,000 X g. Save supernatant.
4. Dilute supernatant 1:4 (i.e. 250 μL of supernatant, 650 μL dH₂O, and 100 μL of Dilution Buffer I). Vortex to mix and analyze as sample (Assay Procedure, step 1). The Ochratoxins concentration contained in samples is then determined by multiplying the ELISA result by the dilution factor of 20. Recoveries were 100-103%.

Wine

1. Measure and add 1.0 mL of wine into a 15 mL plastic centrifuge tube.
2. Add 9.0 mL of 80% methanol-20% DI water-0.1% sodium bicarbonate, vortex thoroughly. Mix using an overhead rotator for 10 minutes.
3. Centrifuge for 5 min at 3,000 X g. Save supernatant.
4. Dilute supernatant 1:7 (i.e. 140 μL of supernatant, 760 μL dH₂O, and 100 μL of Dilution Buffer I). Vortex to mix and analyze as sample (Assay Procedure, step 1). The Ochratoxins concentration contained in samples is then determined by multiplying the ELISA result by the dilution factor of 70. Recoveries were 97-109%.

Pet Food

1. Weigh 0.5 gm of homogenized dried pet food into a 50 mL plastic centrifuge tube.
2. Add 20 mL of a 1:1 MeOH/DI-0.1% bicarbonate, vortex thoroughly. Mix using an overhead rotator for 10 minutes.
3. Centrifuge for 5 minutes at 3,000 X g. Save supernatant.

4. Dilute supernatant 1:5 (i.e. 200 μL of supernatant, 700 μL dH₂O, and 100 μL of Dilution Buffer I. Vortex to mix and analyze as sample (Assay Procedure, step 1). The Ochratoxins concentration contained in samples is then determined by multiplying the ELISA result by the dilution factor of 200. Recoveries were 97-113%.

E. Working Scheme

The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 0	Std 4	Samp 2									
B	Std 0	Std 4	Samp 2									
C	Std 1	Std 5	Samp 3									
D	Std 1	Std 5	Samp 3									
E	Std 2	Samp 1	etc.									
F	Std 2	Samp 1	etc.									
G	Std 3											
H	Std 3											

Std 0-Std 5: Standards

0: 0.025; 0.05; 0.10; 0.20; 0.40 ppb

Samp1, Samp2, etc.: Samples

F. Assay Procedure

1. Add 100 μL of the **standard solutions and samples or sample extracts** into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.
2. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill contents. Incubate the strips for 30 minutes at room temperature.
3. After incubation, remove the covering and vigorously shake the contents of these wells into a sink. Wash the strips **four times** using the 1X washing buffer solution. Use 250 μL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
4. Add 50 μL of **HRP conjugate assay buffer solution** to the individual wells successively using a multi-channel pipette or a stepping pipette.
5. Add 50 μL of **HRP conjugate solution** to the individual wells successively using a multi-channel pipette or a stepping pipette.
6. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill contents. Incubate the strips for 30 minutes at room temperature.
7. After incubation, remove the covering and vigorously shake the contents of these wells into a sink. Wash the strips **three times** using the 1X washing buffer solution. Use 250 μL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
8. Add 150 μL of **substrate (color) solution** to the wells. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Incubate the strips for 20 minutes at room temperature. Protect the strips from direct sunlight.
9. Add 100 μL of **stop solution** to the wells in the same sequence as for the substrate solution.
10. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

G. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs [4-Parameter (preferred) or Logit/Log]. For manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the $\%B/B_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the $\%B/B_0$ for each standard on the vertical linear (y) axis versus the corresponding Ochratoxin concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels in ppb of Ochratoxin by interpolation using the standard curve. Samples showing lower concentrations of Ochratoxin compared to Standard 1 (0.025 ng/mL) should be reported as containing < 0.025 ng/mL. Samples showing a higher concentration than Standard 5 (0.40 ng/mL) must be diluted further with **Dilution Buffer II** provided to obtain accurate results.

H. References

- 1) A.H. Heusser, I. Moeller, B.W. Day, D.R. Dietrich, E. O'Brien. Food and Chem, Toxicology (2007), 45, 827-833.
- 2) A.H. Heusser, D.R. Dietrich, E. O'Brien. Toxicol. In Vitro (2006), 20, 332-341.
- 3) Official Journal of the European Union. Commission Regulation No. 1881/2006.