OCHRATOXIN A UNIVERSAL – QUANTITATIVE ASSAY

Cat. No. 961OCH01LM-96

OCHRATOXIN A

Ochratoxin A is a toxic secondary metabolite produced by several molds of the *Aspergillus* and *Penicillium* genera, including *Aspergillus ochraceus*. Ochratoxin A is a nephrotoxin and carcinogen. In humans, exposure to ochratoxin A has been linked to Balken endemic nephropathy (BEN), a chronic kidney disease associated with tumors of the renal system. Impairment of renal system has also been reported in swine. In turkeys and chickens, symptoms include retarded growth, decreased feed conversion, nephropathy and mortality. Feed refusal has also been observed in turkeys. A decrease in egg production and shell quality was reported in both turkeys and chickens. Ochratoxin A has been frequently detected in human foods and animal feed with the main human bioburden deriving from cereals and grain products, although a wide range of commodities has been found to contain the toxin. These include grains, green and roasted coffee, cocoa, spices and grape derivatives such as raisins, grape juice and wines.

INTENDED USE

The HELICA Ochratoxin A Universal kit is specifically designed for the quantitative determination of ochratoxin A in grains, coffee, cocoa powder and cocoa butter, various spices, alcohol, milk and serum.

FIELD OF USE

Data obtained from Helica™ assays should not be used for human diagnostic or human treatment purposes. Assays are not approved by the United States Food and Drug Administration or any other U.S or non-U.S. regulatory agency for use in human diagnostics or treatment. Helica assays should not be used as the sole basis for assessing the safety of products for release to consumers. The information generated is only to be used in conjunction with the user's regular quality assurance program. Not approved for clinical diagnosis. Use for research and development, quality assurance and quality control under supervision of technically qualified persons.

ASSAY PRINCIPLE

The HELICA Ochratoxin A Universal Assay is a solid phase competitive inhibition enzyme immunoassay. An antibody with high affinity to ochratoxin A is coated to a polystyrene microwell. Toxins are extracted from a ground sample with 70% methanol or 80% acetonitrile and after dilution, added to the appropriate well. Standard or sample is added to the appropriate well and if ochratoxin A is present it will bind to the coated antibody. Subsequently, ochratoxin A bound to horseradish peroxidase (HRP) is added and binds to the antibody not already occupied by ochratoxin A present in the standard or sample. After this incubation period, the contents of the wells are decanted, washed and an HRP substrate is added which develops a blue color in the presence of enzyme. The intensity of the color is directly proportional to the amount of bound conjugate and inversely proportional to the amount of ochratoxin A in the standard or sample. Therefore, as the concentration of ochratoxin A in the sample or standard increases, the intensity of the blue color will decrease. An acid solution is added which changes the chromogen color from blue to yellow. The microwells are measured optically by a microplate reader with an absorbance filter of 450nm (OD_{450}). The optical densities of the samples are compared to the ODs of the kit standards and a result is determined by interpolation from the standard curve.

REAGENTS PROVIDED

1 X Pouch	**Antibody Coated Microwell Plate**		96 wells (12 eight well strips) in a microwell holder coated with a mouse anti-ochratoxin A antibody, *Ready-to-Use.*
1 X Plate	**Mixing Wells**	Green	96 non-coated wells (12 eight well strips) in a microwell holder, *Ready-to-Use.*
6 X Vial	**Ochratoxin A Standards**	Black Cap	1.5mL/vial of ochratoxin A at the following concentrations: 0.0, 0.05, 0.1, 0.2, 0.4, and 0.8ng/mL in 70% methanol, *Ready-to-Use.*
1 X Bottle	**Ochratoxin A HRP-Conjugate**	Green Cap	12mL of ochratoxin A conjugated to peroxidase in buffer with preservative, *Ready-to-Use.*
2 X Bottle	**Assay Diluent**	Brown Cap	2 x 12mL proprietary sample diluent, *Ready-to-Use.*
1 X Bottle	**Substrate Reagent**	Blue Cap	12mL stabilized tetramethylbenzidine (TMB), *Ready-to-Use.*
1 X Bottle	**Stop Solution**	Red Cap	12mL Acidic Solution, *Ready-to-Use*.
1 X Pouch	**Wash Buffer**		PBS with 0.05% Tween20, bring to 1 liter with distilled water and store refrigerated.

MATERIALS REQUIRED BUT NOT PROVIDED

Extraction Procedure

- Grinder sufficient to render sample to particle size of fine instant coffee
- Collection container: Minimum 100mL capacity
- Balance: 20g measuring capability
- Graduated cylinder: 100mL
- Methanol or acetonitrile: 3.5-40mL reagent grade per sample.
- Distilled or deionized water: 1-15mL per sample
- Filter Paper: Whatman #1 or equivalent
- Filter funnel
- Centrifuge

Assay Procedure

- Pipettor with tips: 100µL and 200µL
- Timer
- Wash bottle
- Dilution tubes
- Absorbent paper towels
- Microplate reader with 450nm filter

PRECAUTIONS

1. Bring all reagents to room temperature (19°C - 27°C) before use.
2. Store reagents at 2°C to 8°C, and do not use beyond expiration date(s). Never freeze kit components.
3. Do not return unused reagents back into their original bottles. The assay procedure details volumes required.
4. Adhere to all time and temperature conditions stated in the procedure.
5. Never pipette reagents or samples by mouth.
6. Standards are flammable. Caution should be taken in the use and storage of these reagents.
7. The Stop Solution contains acid. Do not allow to contact skin or eyes. If exposed, flush with water.
8. Consider all materials, containers and devices that are exposed to sample or standards to be contaminated with toxin. Wear protective gloves and safety glasses when using this kit.
9. Dispose of all materials, containers and devices in the appropriate receptacle after use.
10. HRP-labeled conjugate and TMB substrates are photosensitive and are packaged in a protective opaque bottle. Store in the dark and return to storage after use.

KIT SAFETY and WASTE DISPOSAL INSTRUCTIONS

Mycotoxins (aflatoxins, trichothecenes, and others) are well known carcinogens in humans and are thus considered highly toxic. Do not dispose of these materials down the drain. Please note that there is a potential for mycotoxin contamination in or on any of the kit components provided. It is strongly advised that gloves, a lab coat, and safety glasses be worn at all times while handling mycotoxin kits and their respective components.

1. Before conducting the assay, prepare a waste container to act as a receptacle for your kit waste. Eject contaminated pipette tips and all other related materials into this container.
2. Once the assay is completed, the container should be treated with a sufficient amount of 5-6% sodium hypochlorite (NaOCl) to saturate the contents of the container (approximately 1/10th the volume of the container). 5-6% NaOCl will denature the mycotoxins and neutralize the waste, making it safe for disposal. Invert the container several times to thoroughly coat all waste.
3. In the case of an accidental toxin spill, treat the spill surface with 5-6% NaOCl for a minimum of 10 minutes followed by 5% aqueous acetone. Wipe dry with absorbent paper towels.

EXTRACTION PROCEDURE

Note: The sample must be collected according to the appropriate established sampling techniques.

Coffee, cocoa, and spices

1. Grind a representative sample to the particle size of fine instant coffee (50% passes through a 20 mesh screen).
2. Prepare 50mL of extraction solvent. (see section on performance for choice of solvents).
3. Transfer 50mL of extraction solvent to a container and add 10g of the ground sample.
   Note: The ratio of sample to extraction solvent is 1:5 (w/v).
4. Mix by shaking in a sealed container or blender for a minimum of 5 minutes.
5. Allow the particulate matter to settle, then filter 5-10mL of the extract through a Whatman #1 filter paper (or equivalent) and collect the filtrate to be tested. Alternatively, centrifuge a portion of the sample at 3,500rpm for 5 minutes to speed up the separation. Collect the upper layer containing the ochratoxin to be tested.
6. Dilute an aliquot of the extract 1:10 with 70% methanol in distilled water.
7. The sample is now ready. The standards require no pre-dilution before use.
8. Final dilution for use in calculation = 1:50.

Cocoa butter

1. Weigh 1g of cocoa butter into a capped tube.
2. Prepare extraction solvent by adding 1.5mL of distilled or deionized water to 3.5mL of methanol or 1mL of distilled or deionized water to 4mL of acetonitrile (see section on performance for choice of solvents).
3. Transfer 5mL of extraction solvent to the capped tube and place in hot water (50°C-70°C) until the cocoa butter has melted and the solvent has reached the temperature of the water.
   Note: The ratio of sample to extraction solvent is 1:5 (w/v).
4. Mix by shaking the capped tube so that the melted cocoa butter breaks up into small globules to present a greater surface area to the solvent. Maintain the contents of the tube at >37°C during the mixing by returning the tube to the hot water occasionally. Total mixing time should be maintained at 5 minutes.
5. Immediately, pass the sample through a Whatman #1 filter paper (or equivalent) and collect the filtrate to be tested. During the filtration, the cocoa butter may cool and solidify, but enough sample should pass through for testing.
6. Dilute an aliquot of the extract 1:10 with 70% methanol in distilled water.
7. The sample is now ready. The standards require no pre-dilution before use.
8. Final dilution for use in calculation = 1:50

Cereal grains

1. Grind a representative sample to the particle size of fine instant coffee (95% passes through a 20 mesh screen)
2. Prepare extraction solvent (70% methanol) by adding 30mL of distilled or deionized water to 70mL of methanol for each sample to be tested.
3. Transfer 100mL of extraction solvent to a container and add 20g of the ground sample.
   Note: The ratio of sample to extraction solvent is a 1:5 dilution (w/v).
4. Mix by shaking in a sealed container for a minimum of 2 minutes.
5. Allow the particulate matter to settle, then filter 5-10mL of the extract through a Whatman #1 filter paper (or equivalent) and collect the filtrate to be tested.
6. Dilute an aliquot of the extract 1:10 with 70% methanol.
7. The sample is now ready. The standards require no pre-dilution before use.
8. Final dilution for use in calculation = 1:50.

Alcohol and Juice

1. Dilute samples of wine, grape must or juice 1:20 in 70% methanol. Dilute samples of beer 1:2 with absolute methanol.

Serum and Milk

1. To 250µL of sample (serum or milk) add 750µL of absolute methanol. If different volumes are used maintain the sample to methanol ratio at 1:4. Mix vigorously and allow to stand for five minutes at ambient temperature. Centrifuge or filter the sample to clarity and use the supernatant or filtrate for testing.
2. Final dilution for use in calculation = 1:4.

ASSAY PROCEDURE

1. Bring all the reagents to room temperature before use. Reconstitute the PBS-Tween packet by washing out the contents with a gentle stream of distilled water into a 1-Liter container. Q.S. to 1 Liter with distilled water and store refrigerated when not in use.
2. Place one mixing well in a microwell holder for each Standard and Sample to be tested. Place an equal number of Antibody Coated Microtiter Wells in another microwell holder.
3. Dispense 200µL of the Sample Diluent into each mixing well.
4. Using a new pipette tip for each, add 100µL of each standard and prepared sample to appropriate mixing well containing diluent. Mix by priming pipettor at least 3 times.
   Note: Operator must record the location of each Standard and Sample throughout test.
5. Using a new pipette tip for each, transfer 100µL of contents from each mixing well to a corresponding Antibody Coated Microtiter Well. It is recommended that a multi-channel pipettor be used for this step in order to minimize beginning to end variation. Incubate at room temperature for 30 minutes. The mixing wells contain enough solution to run each standard and/or sample in duplicate if so desired.
6. Decant the contents from microwells into a discard basin. Wash the microwells by filling each with PBS-Tween wash buffer, then decanting the wash into a discard basin. Repeat wash for a total of 3 washes.
7. Tap the microwells (face down) on a layer of absorbent towels to remove residual wash buffer.
8. Add 100µL of ochratoxin A-HRP conjugate to each antibody coated well and incubate at room temperature for 30 minutes. Cover to avoid direct light.
9. Repeat steps 6 and 7.
10. Measure the required volume of Substrate Reagent (1mL/strip or 120µL/well) and place in a separate container. Add 100µL to each microwell. Incubate at room temperature for 10 minutes. Cover to avoid direct light.
11. Measure the required volume of Stop Solution (1mL/strip or 120µL/well) and place in a separate container. Add 100µL in the same sequence and at the same pace as the Substrate Reagent was added.

12. Read the optical density (OD) of each microwell with a microtiter plate reader using a 450nm filter. Record the optical density (OD) of each microwell.
    Setting the zero standard as 100% binding (Bo), calculate % binding (%B) for each standard and sample as a percentage of the zero binding (%B/Bo).

Note: It is the nature of immunoassay curves that they become flat at the extreme low and high values. Extrapolation to values beyond the lowest and highest point on the standard curve will lead to imprecise and inaccurate results.

INTERPRETATION OF RESULTS

Construct a dose-response curve using either the unmodified OD values or the OD values expressed as a percentage (%B/Bo) of the OD of the zero (0.0) standard against the ochratoxin A content of the standard. Unknowns are measured by interpolation from the standard curve.

The information contained on the label of each standard vial refers to the contents of that vial. However, the sample has been diluted by extraction solvent as instructed in the EXTRACTION PROCEDURE, so the level of ochratoxin shown by the standard must be multiplied by the total dilution factor in order to indicate the ng per gram (ppb) of the commodity as follows:

Standard (ng/mL)	**Cocoa, cocoa butter, coffee spices, grains (ppb) 1:50**	**Wine, grape must, juice (ppb) 1:20**	**Serum and milk (ppb) 1:4**	**Beer (ppb) 1:2**
0	0	0	0	0
0.05	2.5	1	0.2	0.1
0.1	5	2	0.4	0.2
0.2	10	4	0.8	0.4
0.4	20	8	1.6	0.8
0.8	40	16	3.2	1.6

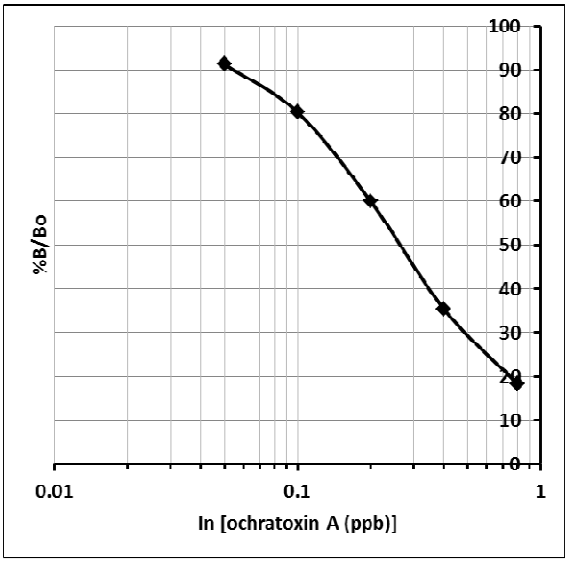
If a sample contains ochratoxin A at a greater concentration than the highest standard, it should be diluted appropriately in extraction solvent and retested. The extra dilution step should be taken into consideration when expressing the final result.

ASSAY CHARACTERISTICS

Data from twenty-one consecutive standard curves gave the following results:

Standard (ng/mL)	**Concentration in commodity (1:50)**	**%B/B_0**	**%CV**
0	0	100.0	-
0.05	2.5	91.3	4.0
0.1	5	80.4	5.7
0.2	10	60.0	7.6
0.4	20	35.4	9.7
0.8	40	18.4	17.8

The below figure is a representative standard curve for ochratoxin A based on the data table from the previous page.



Green coffee determined to be <1ppb by HPLC was obtained from Trilogy Labs (etc.). The remainder of the commodities used to determine the performance parameters of the assay were purchased as consumer products and were not further analyzed by HPLC. Each commodity was extracted in three different solvents: Solvent 1: 70% methanol in 1% sodium bicarbonate; Solvent 2: 70% methanol in distilled water; Solvent 3: 80% acetonitrile in distilled water. Each extract was diluted 10:1 in 70% methanol in distilled water as described in 'Extraction Procedure'. Each diluted sample was assayed within 12 replicates against the zero standard. Results are given below:

Solvent 1: n=12

	Mean %B/B0	%CV	ppb	Mean-2 standard deviations (ppb)
Green Coffee	95.7	2.0	<1	<1
Roast Coffee	99.3	1.1	<1	<1
Instant Coffee	92.3	1.0	<1	<1
Cocoa Powder	93.0	1.4	<1	<1
Cocoa Butter	99.3	3.4	<1	<1
Paprika	99.9	1.8	<1	<1
Chili Powder	100.5	1.6	<1	<1

Solvent 2: n=12

	Mean %B/B0	%CV	ppb	Mean-2 standard deviations (ppb)
Green Coffee	94.9	2.5	<1	1.1
Roast Coffee	99.7	2.3	<1	<1
Instant Coffee	90.5	2.0	<1	1.1
Cocoa Powder	90.7	2.2	<1	1.2
Cocoa Butter	99.6	2.7	<1	<1
Paprika	97.7	1.5	<1	<1
Chili Powder	93.2	2.3	<1	<1

Solvent 3: n=12

	Mean %B/B0	%CV	ppb	Mean-2 standard deviations (ppb)
Green Coffee	95.4	1.1	<1	<1
Roast Coffee	94.9	2.8	<1	<1
Instant Coffee	92.3	2.1	<1	1.0
Cocoa Powder	90.7	2.6	1.0	1.3
Cocoa Butter	101.7	2.6	<1	<1
Paprika	96.4	1.7	<1	<1
Chili Powder	94.2	1.4	<1	<1

In order to determine the extraction efficiency of the three solvents, 1g of each commodity was spiked within 5ppb of ochratoxin A in absolute methanol, dried overnight and then extracted as in 'Extraction Procedure'. In the case of cocoa butter the solid, waxy substance was scraped into tiny slivers, spiked and after drying was melted in hot water and re-solidified so that the added ochratoxin A became incorporated into a solid homogeneous whole to simulate more closely the naturally occurring situation. Spiking material was diluted into 5mL of extraction solvent and compared to the 5mL of commodity extract as continued. Extractions were performed three times for each commodity. Results are presented below:

Solvent 1:

	Recovery 1 (%)	Recovery 2 (%)	Recovery 3 (%)
Green Coffee	83	92	85
Roast Coffee	79	84	79
Instant Coffee	79	81	73
Cocoa Powder	94	94	92
Cocoa Butter	90	87	91
Paprika	79	81	73
Chili Powder	87	96	97

Solvent 2:

	Recovery 1 (%)	Recovery 2 (%)	Recovery 3 (%)
Green Coffee	93	79	83
Roast Coffee	75	79	78
Instant Coffee	75	74	89
Cocoa Powder	89	108	113
Cocoa Butter	101	101	98
Paprika	77	102	96
Chili Powder	98	79	81

Solvent 3:

	Recovery 1 (%)	Recovery 2 (%)	Recovery 3(%)
Green Coffee	99	96	90
Roast Coffee	91	97	92
Instant Coffee	107	103	98
Cocoa Powder	110	108	108
Cocoa Butter	97	104	100
Paprika	102	104	109
Chili Powder	101	102	107

It appears that Solvent 3, 80% acetonitrile in distilled water, is the more generally applicable solvent of choice, though methanol works well within the cocoa products. Ochratoxin A spiked directly into solvent multiple times and assayed as control in the recovery experiments measured 4.86 ± 0.39ppb (CV= 8.0%, n= 27).

Recoveries of ochratoxin A from certified reference corn samples were as follows based on five independent experiments using 70% methanol (n=5):

Corn Reference Sample [ppb]	Recovery [%]	Repeatability [%CV]
18	101	8.9
4.8	104	10.6
2.9	95	10.2

Alcohol was also tested. All commodities were grown and processed in California except for the beer which was brewed in Belgium.

Comparing the commodities to the zero standard (70% methanol) in four assays in duplicate gave the following results:

	OD	SD	CV%
Standard Zero	1.918	0.09	4.7
Red Wine (Merlot)	1.922	0.09	4.7

Standard Zero	1.847	0.11	6.0
White Wine (Chardonnay)	1.817	0.11	6.1

Standard Zero	1.926	0.11	5.7
Port	1.909	0.13	6.8

Standard Zero	1.945	0.11	5.7
Sherry	1.955	0.05	2.6

Standard Zero	2.030	0.12	5.9
Red Grape Must	2.045	0.09	4.4

Standard Zero	1.827	0.07	3.8
Red Grape Juice	1.819	0.07	3.8

Standard Zero	1.870	0.09	4.8
Beer	1.841	0.11	6.0

Having shown that none of the commodities contained Ochratoxin A, each was spiked with Ochratoxin A, at levels of 0.0,0.4,1.0,2.0, 4.0, and 8.0 ng/mL and the standard solvent (70% methanol) was similarly spiked. Beer was spiked at 0.0, 0.04, 0.1, 0.2, 0.4, and 0.8 ng/mL. All samples were diluted 1:20 with 70% methanol except the beer which was diluted 1:2 in absolute methanol and assayed as described above (In the kit as presented the standards are pre-diluted and should be used without further dilution). Recoveries for each commodity with reference to the standards are given below.

Standard (ng/mL)	Red Wine%	White Wine%	Port %	Sherry %	Must %	Juice %	Beer %
0.05	104	98	102	104	101	72	112
0.10	100	92	98	105	99	80	115
0.20	103	110	98	93	98	93	120
0.40	110	99	108	101	91	95	113

The results demonstrate that the Helica Biosystems Quantitative Ochratoxin A assay can be used to measure Ochratoxin A in a wide variety of alcoholic and non-alcoholic beverages.

Biological samples were also tested on the Ochratoxin A Universal ELISA. The following samples were tested in the assay: 1. Charcoal stripped normal human serum. 2. Charcoal stripped normal pig serum. 3. Human Colostrum/milk 4. Full-fat cow milk. Each was measured with 12 replicates and compared to the zero standard.

	% B_0 sample	%B_0 sample <2SD	CV%	ng/mL
Human Serum	99.7	96.3	1.7	<0.08
Pig Serum	100.2	97.4	1.4	<0.08
Human Milk	92.3	89.3	1.6	<0.08
Cow's Milk	91.1	88.9	1.2	<0.08

Results indicate that these samples are negative (<0.08ng/mL) for ochratoxin A. These serum and milk samples were spiked with approximately 0.2ng/mL Ochratoxin A and after equilibrating overnight, were extracted and assayed as described. Extraction was performed three times for each sample. PBS was spiked and extracted in the same manner as control.

	Recovery 1(%)	Recovery 2(%)	Recovery 3(%)	Recovery Mean(%)
Human Serum	102	102	104	103
Pig Serum	100	94	96	97
Human Milk	96	110	95	100
Cow's Milk	114	116	113	114

PBS control measured 0.214 ± 0.011 ng/mL; CV = 5.1%, n= 8.
The consistently higher than 100% recovery values for the cow's milk sample would indicate an intrinsic 0.0 to 0.08ng/mL level of Ochratoxin A.