Helica® Ochratoxin A Universal ELISA

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Helica® Ochratoxin A Universal ELISA

For the quantitative determination of ochratoxin A in grains, coffee, cocoa powder and cocoa butter, various spices, alcohol, milk and serum.

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Introduction – 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 Balkan endemic nephropathy (BEN), a chronic kidney disease associated with tumors of the renal system. 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. Impairment of renal system has also been reported in swine. 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

Hygiena's Helica® Mycotoxin ELISA kits are user-friendly, cost-effective kits for the detection of mycotoxins in a wide range of commodities including animal feeds, grains, corn and animal urine, designed to protect humans and animals from dangerous side effects of mycotoxins.

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

Data obtained from 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 US or non-US regulatory agency for use in human diagnostics or treatment. 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 the supervision of technically qualified persons.

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Principle of the Method

The Helica Ochratoxin A Universal ELISA 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 450 nm (OD450). 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.

Kit Contents

Package/ Number	Component	Description
1X 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*.
1X Plate	Dilution plate	96 non-coated wells (12 eight-well strips) in a microwell holder, *Ready-to-Use*.(Mixing wells)
6X Vials	Standards	1.5 mL/vial of ochratoxin A at the following concentrations: 0.0, 0.05, 0.1, 0.2, 0.4, and 0.8 ng/mL in 70% methanol, *Ready-To-Use*.
1X Bottle	Conjugate	12 mL of ochratoxin A conjugated to peroxidase in buffer with preservative, *Ready-to-Use*.
2X Bottles	Assay Diluent	2 x 12 mL proprietary sample diluent, Ready-to-Use.
1X Bottle	Substrate	12 mL stabilized tetramethylbenzidine (TMB), *Ready-to-Use*.
1X Bottle	Stop solution	12 mL acidic solution, *Ready-to-Use*.
1X Pouch	PBS-T powder	PBS with 0.05% Tween® 20*, bring to 1 Liter with distilled water and store refrigerated. (Wash buffer)

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Materials Required But Not Provided

- Grinder sufficient to render sample to a particle size of fine instant coffee
- Collection container with minimum 100 mL capacity
- Balance with 20 g measuring capability
- Graduated cylinder: 100 mL
- Methanol or acetonitrile: 3.5-40 mL, reagent grade per sample
- Distilled or deionized water: 1-15 mL per sample
- Filter paper: Whatman #1 or equivalent
- Filter funnel
- Centrifuge
- Pipettor with tips: 100 µL and 200 µL
- Timer
- Wash bottle
- Dilution tubes
- Absorbent paper towels
- Microplate reader with 450 nm filter

Storage and Shelf Life

- Store reagents at 2 to 8 °C. Do not freeze.
- Reagents should be used by the expiration date stamped on the individual labels.
- HRP-labeled conjugate and TMB-substrates are photosensitive and are packaged in a light-protective opaque bottle. Store in the dark and return to storage after use.

Precautions and Waste Disposal

General Precautions:

- Bring all reagents to room temperature (19 to 25 °C) before use.
- Do not interchange reagents between kits of different lot numbers.
- Do not use solutions if cloudy or precipitate is present.
- Do not return unused reagents to their original bottles. The assay procedure details volumes required.
- Adhere to all time and temperature conditions stated in the procedure.
- During the sample extraction, avoid cross-contamination.
- Devices, such as a blender, must be cleaned after each sample preparation.
- Samples tested should have a pH of 7.0 (± 1.0). Excessive alkaline or acidic conditions may affect the test results.

Safety Precautions:

Mycotoxins (aflatoxins, trichothecenes and others) are well-known human carcinogens and are considered highly toxic. Because mycotoxins can cause human illness, appropriate safety precautions must be taken and personal protective equipment worn when handling samples, reagents, glassware and other supplies and equipment that potentially could be contaminated with mycotoxins.

- Before using this assay, please review the Safety Data Sheet(s) available at www.hygiena.com.
- Refer to your site practices for safe handling of materials.
- It is strongly advised that protective gloves, a lab coat and safety glasses be worn at all times while handling mycotoxin kits and their respective components. Consider all materials, containers and devices that are exposed to samples or standards to be contaminated with mycotoxins.
- Never pipette reagents or samples by mouth.
- Standards are flammable. Caution should be taken in the use and storage of these reagents.
- The stop solution contains sulfuric acid, which is corrosive. Please refer to the SDS. Do not allow to contact skin or eyes. If exposed, flush with water.

Disposal:

Decontaminate materials and dispose of waste per your site practices and as required by local regulations. 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.

- Dispose of all materials, containers and devices in the appropriate receptacle after use.
  Before conducting the assay, prepare a waste container to act as a receptacle for your kit waste.
  Eject contaminated pipette tips and discard all other related materials into this container.
- 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). The NaOCl will denature the mycotoxins and neutralize the waste, making it safe for disposal. Invert the container several times to thoroughly coat all waste.
- 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.

Preparation of Samples

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

Extraction Procedure

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 50 mL of extraction solvent. (see the section on performance for choice of solvents).
3. Transfer 50 mL of extraction solvent to a container and add 10 g 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 5-10 mL 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 3,500 rpm for 5 minutes to speed up the separation.
6. Collect the upper layer containing the ochratoxin to be tested.
7. Dilute an aliquot of the extract 1:10 with 70% methanol in distilled water.
8. The sample is now ready for testing.
9. The 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 1.5 mL of distilled or deionized water to 3.5 mL of methanol or 1 mL of distilled or deionized water to 4 mL of acetonitrile (see section on performance for choice of solvents).
3. Transfer 5 mL 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 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 for testing.
8. The 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 30 mL of distilled or deionized water to 70 mL of methanol for each sample to be tested.
3. Transfer 100 mL of extraction solvent to a container and add 20 g 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 5-10 mL 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 for testing.
8. The 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.
2. The final dilutions for use in calculation = **1:20** (wine, grape must or juice) or **1:4** (beer).

Serum and Milk

1. To 250 µL of sample (serum or milk), add 750 µL 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.
2. Centrifuge or filter the sample to clarify and use the supernatant or filtrate for testing.
3. The final dilution for use in calculation = **1:4**

Assay Procedure

Note: It is recommended that a multi-channel pipettor be utilized to perform the assay. If a single channel pipettor is used, it is recommended to run no more than a total of 16 samples and standards (2 test strips).

1. Bring all the reagents to room temperature before use. Prepare wash buffer by reconstituting the PBS-T powder packet by washing out the contents with a gentle stream of distilled or deionized water into a 1-Liter container. Fill 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. If running single well, adjust volumes accordingly. Return unused wells to the foil pouch with desiccant and reseal.
3. Mix each reagent by swirling the reagent bottle prior to use.
4. Dispense 200 µL of the sample diluent into each mixing well.
5. Using a new pipette tip for each, add 100 µL of each standard and prepared sample to appropriate mixing well containing diluent. Mix by priming pipettor at least three (3) times. *Note: The operator must record the location of each Standard and Sample throughout test.*
6. Using a new pipette tip for each, transfer 100 µL 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.
   Note: The mixing wells contain enough solution to run each standard and/or sample in duplicate (recommended). If running each standard or sample in singlets or if more replicates are needed, the volumes of assay diluent and sample/ standard should be scaled accordingly.
7. Decant the contents from microwells into a discard basin. Wash the microwells by filling each with PBS-T wash buffer, then decanting the wash into a discard basin. Repeat wash for a total of three (3) washes.
8. Tap the microwells (face down) on a layer of absorbent towels to remove residual wash buffer.
9. Add 100 µL of ochratoxin A-HRP conjugate to each antibody-coated well and incubate at room temperature for 30 minutes. Cover to avoid direct light.
10. Repeat steps 6 and 7.
11. Measure the required volume of Substrate Reagent (1 mL/strip or 120 µL/well) and place into a separate container. Add 100 µL to each microwell. Incubate at room temperature for 10 minutes. Cover to avoid direct light.
12. Measure the required volume of Stop Solution (1 mL/strip or 120 µL/well) and place into a separate container. Add 100 µL in the same sequence and at the same pace as the Substrate Reagent was added.
13. 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.

14. Setting the zero standard as 100% binding (B_0), calculate % binding (%B) for each standard and sample as a percentage of the zero binding (%B/B_0).

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 the OD values expressed as a percentage (%B/B_o) of the OD of the zero (0.0 ng/mL) 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 (ng/mL) 1:20	Serum and Milk (ng/mL) 1:4	Beer (ng/mL) 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

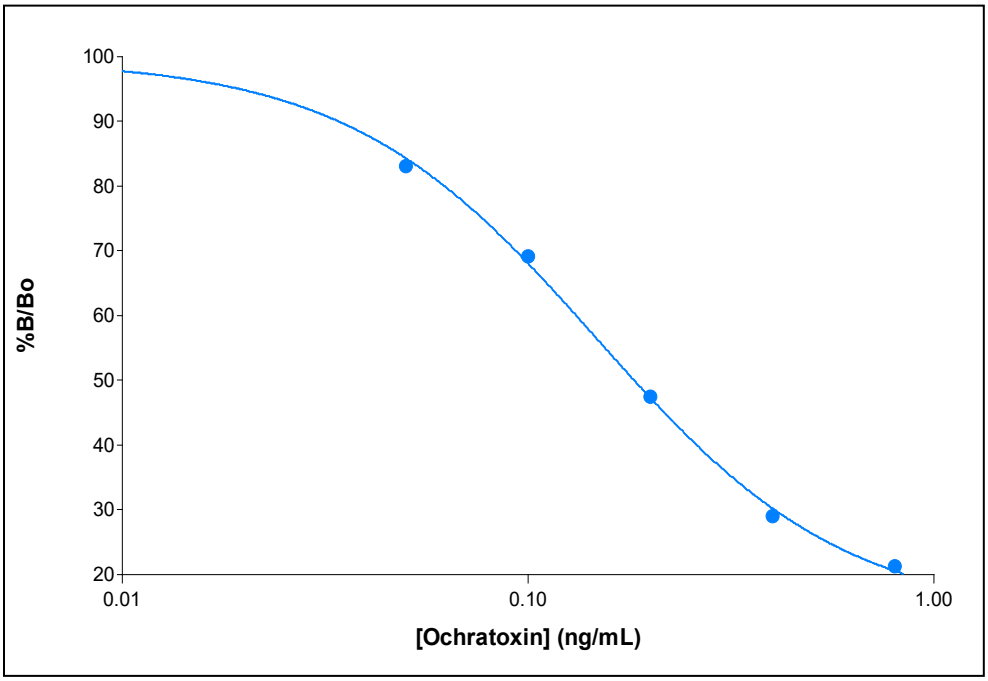
If a sample contains ochratoxin A at a greater concentration than the highest standard, it should be diluted appropriately in extraction solvent and retested. The extra dilution step should be taken into consideration when expressing the final result.

Assay Characteristics

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

Standard (ng/mL)	% B/B_0	% CV
0	100.0	-
0.05	91.3	4.0
0.1	80.4	5.7
0.2	60.0	7.6
0.4	35.4	9.7
0.8	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 contain <1 ppb ochratoxin A 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 with an average of 12 replicates against the zero standard. Results are given below:

Solvent 1, n=12

Commodity	Mean %B/B_0	% 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

Commodity	Mean %B/B_0	% 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

Commodity	Mean %B/B_0	% 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, 1 g of each commodity was spiked with 5 ppb 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 5 mL of extraction solvent and compared to the 5 mL of commodity extract as continued. Extractions were performed three times for each commodity. Results are presented below:

Solvent 1

Commodity	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

Commodity	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

Commodity	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 with the cocoa products. Ochratoxin A spiked directly into solvent multiple times and assayed as a control in the recovery experiments measured 4.86 ± 0.39 ppb (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 average results:

Sample	OD	Std Dev	%CV
Standard Zero	1.918	0.09	4.7
Red Wine (Merlot)	1.922	0.09	4.7

Sample	OD	Std Dev	%CV
Standard Zero	1.847	0.11	6.0
White Wine (Chardonnay)	1.817	0.11	6.1

Sample	OD	Std Dev	%CV
Standard Zero	1.926	0.11	5.7
Port	1.909	0.13	6.8

Sample	OD	Std Dev	%CV
Standard Zero	1.945	0.11	5.7
Sherry	1.955	0.05	2.6

Sample	OD	Std Dev	%CV
Standard Zero	2.030	0.12	5.9
Red Grape Must	2.045	0.09	4.4

Sample	OD	Std Dev	%CV
Standard Zero	1.827	0.07	3.8
Red Grape Juice	1.819	0.07	3.8

Sample	OD	Std Dev	%CV
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
0.1	100	92	98	105	99	80	115
0.2	103	110	98	93	98	93	120
0.4	110	99	108	101	91	95	113

The results demonstrate that the Helica 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's Milk. Each was measured using 12 replicates and compared to the zero standard.

Commodity	% B_0 Sample	% B_0 Sample <2 Std. Dev	% 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 (<0.08 ng/mL) for ochratoxin A. These serum and milk samples were spiked with approximately 0.2 ng/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 the control.

Commodity	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.

Technical Assistance

For questions or comments, please contact your local distributor. You can also email techsupport@hygiena.com, visit our Contact Us page for regional phone numbers or request technical support at https://www.hygiena.com/hygiena/technical-support-request.html.