OCHRATOXIN A IN WINE - QUALITATIVE ASSAY

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Cat No. 981OCH01W-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. Ochratoxin A has been frequently detected in human foods and animal feed with the main human bioburden deriving form cereals and grain products, although a wide range of commodities has been found to contain the toxin. These include green and roasted coffee, cocoa, spices and grape derivatives such as raisins, grape juice and wines.

INTENDED USE

The HELICA Ochratoxin A in Wine Assay has been specifically designed for the qualitative or semi-quantitative estimation of ochratoxin A in liquid vine products from grape must to fortified wines, around the EU limit of 2ppb (µg/L). Samples whose visual estimation lies between 1ppb and 2ppb should be further evaluated by an alternative method such as HPLC or quantitative ELISA. This should facilitate in process control of wine production and provide a quick and inexpensive check on wines from emerging producers.

ASSAY PRINCIPLE

The HELICA Ochratoxin A in Wine Assay is a solid phase competitive inhibition enzyme immunoassay. An antibody with high affinity to ochratoxin A is coated to a polystyrene microwell. 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 horse-radish 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 a 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. A solution containing a red dye is added to stop the enzyme reaction and simultaneously causes a color change leaving the bluest wells deep blue/purple and the lightest wells a mauve/pink, thus facilitating a visual qualitative estimate of ochratoxin A in the sample by comparing to the color of the standards.

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**	Red	96 non-coated wells (12 eight well strips) in a microwell holder, *Ready-to-Use.*
3 X Vial	**Ochratoxin A Standards**	Black Cap	1.5mL/vial of ochratoxin A at the following concentrations: 0.0, 0.3, and 0.6ng/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

- Collection container: Minimum 100mL capacity
- Balance: 20g measuring capability
- Graduated cylinder: 100mL
- Methanol or acetonitrile: 7 or 80mL reagent grade per sample
- Distilled or deionized water: 3 or 20mL per sample
- Centrifuge

Assay Procedure

- Pipettor with tips: 100µL and 200µL
- Timer
- Wash bottle
- Dilution tubes
- Absorbent paper towels
- Microplate reader with 650nm filter (optional)

PRECAUTIONS

1. Bring all reagents to room temperature (19ºC - 27ºC) before use.
2. Store reagents at 2ºC 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.

Wine, Port, Sherry, Grape must, and Grape juice

1. Pipette 3mL of sample into a disposable 10mL tube.
2. Add 7mL of absolute methanol, cap the tube and shake vigorously to mix.
   Note: The ratio of sample to solvent is 1:3.33 (v/v).
3. The sample is now ready. The standards require no pre-dilution before use.
4. Final dilution for use in interpretation = 1:3.33

Licorice

1. Prepare extraction solvent (80% acetonitrile) by adding 20mL of distilled water to 80mL of acetonitrile for each sample to be tested.
2. Transfer 100mL of 80% acetonitrile to a container and add 10g of sample.
   Note: The ratio of sample to solvent is 1:10 (w/v).
3. Mix by shaking in a sealed container for a minimum of 5 minutes.
4. Allow the acetonitrile and licorice layers to separate. Alternatively, centrifuge a portion of the sample at 3,500rpm for 5 minutes to speed the separation. Collect the upper layer containing the ochratoxin to be tested.
5. Dilute an aliquot of the extract 1:10 with 70% methanol.
6. The sample is now ready. The standards require no pre-dilution before use.
7. Final dilution for use in interpretation = 1:100

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 20 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 10 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. Compare the color of the sample wells to the standards with the appropriate final dilution factor to determine compliance with local and internationally accepted limits for ochratoxin A. Alternatively, a permanent record can be made by reading the OD of the wells at 650nm.

INTERPRETATION OF RESULTS

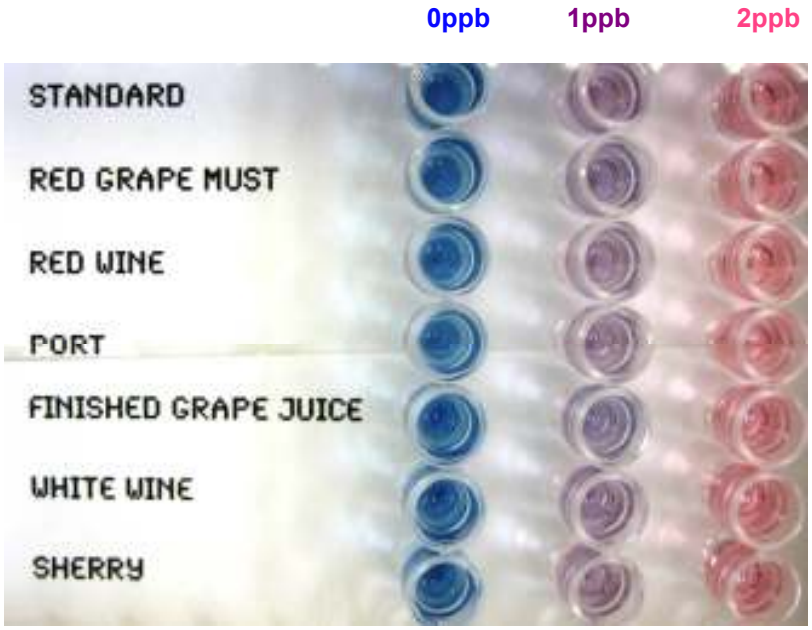
The information contained on the label of each standard vial refers to the contents of that vial. However, the vine samples have been diluted at a 3.33:1 ratio with absolute methanol in the EXTRACTION PROCEDURE, and so the level of ochratoxin shown by the standard must be multiplied by 3.33 in order to indicate the ng per mL (ppb) of the commodity. Additionally, the licorice sample has been diluted at a 10:1 ratio by extraction solvent as instructed in the EXTRACTION PROCEDURE and also 10:1 in 70% methanol and so the level of ochratoxin shown by the standard must be multiplied by 100 in order to indicate the ng per gram (ppb) of the commodity as follows:

	Wine, Port, Sherry, Grape Must, and Grape Juice	Licorice
standard ng/mL	**commodity (ppb) 1:3.33**	**commodity (ppb) 1:100**
0	0	0
0.3	1	30
0.6	2	60

Compare the color of the unknown sample to the color of the standards to determine if the unknown sample is above or below the desired cut-off value.

ASSAY CHARACTERISTICS

Typical assay results are shown for vine products ranging from must to fortified wines spiked with 1ppb or 2ppb ochratoxin A. There is a clear distinction between 0ppb, 1ppb, and 2ppb.



The HELICA Ochratoxin A in Wine Assay is intended primarily as a qualitative/semi-quantitative assay with a visual end-point. The inter-assay and intra-assay variability of 0ppb, 1ppb, and 2ppb ochratoxin A that were spiked into the commodities with respect to the methanol standards was assessed using a standard acid stop method with subsequent OD measurements of 450nm (i.e. yellow endpoint). The following data were obtained:

INTRA-ASSAY COMPARISON

Data from eight consecutive standard curves performed alongside eight replicates of spiked commodities gave the following results:

	Standard		Grape Must	
ochratoxin A (ppb)	Mean OD	%CV	Mean OD	%CV
0	1.854	4.2	1.850	4.4
1	0.874	5.7	0.959	3.9
2	0.371	8.9	0.413	5.3

	Standard		Red Wine	
ochratoxin A (ppb)	Mean OD	%CV	Mean OD	%CV
0	1.881	4.0	1.870	4.7
1	1.011	3.1	1.109	3.3
2	0.427	4.9	0.451	3.1

	Standard		White Wine	
ochratoxin A (ppb)	Mean OD	%CV	Mean OD	%CV
0	1.515	2.8	1.487	3.8
1	0.702	2.8	0.756	4.1
2	0.278	6.5	0.322	6.5

	Standard		Grape Juice	
ochratoxin A (ppb)	Mean OD	%CV	Mean OD	%CV
0	2.035	1.2	2.009	5.5
1	1.001	3.0	1.044	3.4
2	0.447	6.3	0.366	7.1

INTER-ASSAY COMPARISON

Data from eight independent assays performed alongside spiked commodities gave the following results. Spiked samples were calculated as a percentage of the 0ppb, 1ppb, and 2ppb methanol standards.

	0ppb		1ppb		2ppb	
Commodity	% Standard	%CV	% Standard	%CV	% Standard	%CV
Grape Must	98.5	3.2	116.2	4.8	102.6	8.5
Red Wine	97.2	2.8	99.5	3.0	103.7	8.5
White Wine	97.2	2.3	100.3	7.0	98.5	5.8
Grape Juice	95.5	2.2	107.5	5.1	85.8	5.9