helica
biosystems inc

QUANTITATIVE ASSAY FOR OCHRATOXIN A IN COFFEE, COCOA, AND SPICES (96-well kit) **CAT. NO. 961OCH01COF-Qual**

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 (Assessment of Dietary Intake of Ochratoxin A by the Population of EU Member States: Report of Experts Participating in Task 3.2.7, Jan 2002).

INTENDED USE

The Helica Ochratoxin A Coffee, Cocoa, and Spices has been specifically designed for the quantitative determination of Ochratoxin A in green, roast, and instant (soluble) coffee; cocoa powder and cocoa butter and various spices in the range of 1-20 ppb (μg/kg).

ASSAY PRINCIPLE

The HELICA Ochratoxin A Coffee, Cocoa, and Spices is a solid phase direct enzyme immunoassay. An antibody with high affinity to Ochratoxin A is coated onto polystyrene microwells. 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 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. The reaction is stopped by the addition of an acid solution which causes the blue color to change to yellow.

MATERIALS SUPPLIED

1 pouch:	Antibody coated microwells	96 wells (12 x 8-well strips) in a microwell holder
		coated with a mouse anti-ochratoxin A antibody
1 plate:	Mixing wells	96 wells non-coated (12x8-well strips) in a microwell holder. The wells are color coded red.
6 vials:	Ochratoxin A Standards *	1.5 mL/vial of ochratoxin A at the following concentrations
		0.0, 0.02, 0.05, 0.1, 0.2, 0.4 ng/mL in 70% methanol (see page 3) *
2 bottles:	Assay diluent	2 x12 mL proprietary assay diluent
1 bottle:	Ochratoxin A HRP-conjugate	12 mL ochratoxin A conjugated to HRP in buffer with preservative
1 bottle:	Substrate Reagent	12 mL stabilized TMB
1 bottle:	Stop Solution	12 mL acidic stop solution
1 pouch:	Wash buffer (PBS-T)	PBS WITH 0.05% Tween20®, bring to 1 liter with distilled water and store refrigerated

MATERIALS REQUIRED BUT NOT PROVIDED

Pipettor with tips: 100 µl and 200 µl
Absolute methanol
1 acetonitrile
Wash bottle
Absorbent paper towels
Timer

PRECAUTIONS

1. Bring all reagents to room temperature (19º - 27ºC) before use.
2. Store reagents at 2 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. 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.
8. Dispose of all materials, containers and devices in the appropriate receptacle after use.

SAMPLE PREPARATION

1. In the case of coffee, cocoa powder and spices the sample should be ground so that 50% would pass through a 20 mesh screen.
2. Weigh a portion into a container and add a 5:1 ratio of the preferred solvent, eg. 50 mL to 10 gm. (se section on performance parameters for choice of solvents).
3. Mix vigorously for 5 minutes.
4. Filter or centrifuge a small quantity of the supernatant to clarify.
5. Cocoa butter presents a special case. Weigh 1 gm of cocoa butter into a capped tube. Add 5 mLs of solvent and place in hot water (50°-70°C) until the cocoa butter has melted and the solvent has reached the temperature of the water. Mix the contents of the tube vigorously 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. Proceed as in step 4 above.
6. Dilute each clarified extract 10:1 with 70% methanol in distilled water. Eg. 100 µL extract plus 900 µL 70% methanol.
7. The sample is now ready for assay.

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 200 µl of the assay diluent into each mixing well.
4. 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 3 times.
   Note: Operator must record the location of each Standard and Sample throughout test.
5. 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. 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 water.
8. Add 100 µl of conjugate to each antibody coated well and incubate at ambient temperature for 30 minutes.
9. Repeat steps 6 and 7.
10. Measure the required volume of Substrate Reagent (1 mL/strip or 120 µl/well) and place in a separate container. Add 100 µl to each microwell. Incubate at ambient temperature for 10 minutes
11. Measure the required volume of Stop Solution (1 mL/strip or 120 µl/well) and place in a separate container. Add 100 µl in the same sequence and at the same pace as the Substrate was added.
12. Compare the color of the sample wells to the standards to determine compliance with local and internationally accepted limits for Ochratoxin A. For ground green or roasted coffee, 5ppb and for liquid coffee, 10ppb. Please see end page 5 for end point color indicators. Alternatively a permanent record can be made by reading the OD of the wells at 650 nm.
13. Alternaitively, construct a dose-response standard curve of optical density (OD) against Ochratoxin A content. Sample unknowns are measured by interpolation from the standard curve. If a sample is higher than the highest standard, it should be further diluted in 70% methanol and re-tested. The added dilution factor should be taken into account when expressing the result.

ASSAY CHARACTERISTICS

The values for Ochratoxin A on the standards refer to the contents of the vial. As the commodity being tested has been extracted within a 5;1 ratio of solvent and subsequently diluted another 10:1 this translates to a value in the commodity 50 fold higher than the standards as follows:

Standard (ng/mL)	Commodity ppb (µg/kg)
0.0	0.0
0.02	1.0
0.05	2.5
0.10	5.0
0.20	10.0
0.40	20.0

PERFORMANCE PARAMETERS

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% an acetonitrile in distilled water. Each extract was dilted 10:1 in 70% methanol in distilled water as described in 'Sample Preparation'. Each diluted sample was assayed within 12 repliates against the zero and .02 ng/mL 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

RECOVERY DATA

In order to determine the extraction efficiency of the three solvents, I gm of commodities were spiked within 5 ppb of Ochratoxin A in absolute methanol, dried overnight and then extracted as in 'Sample Preparation'. In the case of cocoa butter the solid, waxy substance was scarped into tiny slivers, spiked and after drying was melted in hot water and re-solidified so that the added Ochratoxin became incorporated into a solid homogeneous whole to simulate more closely the naturally occurring situation. Spiking material was diluted into 5 mLs of extraction solvent and compared to the 5 mLs 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, is the more generally applicable solvent of choice, though methanol works well within the cocoa products. Ochratoxin A spiked direcly into solvent multiple times and assayed as control in the recovery experiments measured 4.86+/-.39 ppb (CV= 8.0%, n= 27).

END POINT COLOR INDICATORS