



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

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 from 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 |
| 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, acetonitrile
 Wash bottle
 Absorbent paper towels
 Timer

PRECAUTIONS

1. Bring all reagents to room temperature (19° - 27°C) 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 mLs to 10 gm. (see 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 mL 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. E.g. 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.
5. Note: Operator must record the location of each Standard and Sample throughout test. 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.

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7. Tap the microwells (face down) on a layer of absorbent towels to remove residual water.
8. Add 100 μ l 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 μ l/well) and place in a separate container. Add 100 μ l to each microwell. Incubate at ambient temperature for 10 minutes.
11. Measure the required volume of Stop Solution (1 mL/strip or 120 μ l/well) and place in a separate container. Add 100 μ l in the same sequence and at the same pace as the Substrate was added.
12. 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 with 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 (μ g/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 Analytical Laboratory (Washington, MO, USA). 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** = 80% 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 'Sample Preparation'. Each diluted sample was assayed with 12 replicates. Results are given below:

Solvent 1 (70% methanol in 1% sodium bicarbonate)

| | Mean B/Bo % | CV% (n=12) | ppb | Mean minus 2SD 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 (80% methanol in distilled water)

| | Mean B/Bo % | CV% (n=12) | ppb | Mean minus 2SD 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 (80% acetonitrile in distilled water)

| | Mean B/Bo % | CV% (n=12) | ppb | Mean minus 2SD 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, 1 gm of commodities were spiked with 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 scraped into tiny slivers, and 1 gm placed in a 10 mL capped tube. After spiking and drying, the material was melted in hot water. It was then cooled to re-solidify the cocoa butter 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 control. 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 with the cocoa products. Ochratoxin A spiked directly into solvents 2 and 3 multiple times and assayed as control in the recovery experiments measured 4.86 +/- 0.39 ppb (CV= 8.0%, n= 27).