AOAC Official Method 2000.09
Ochratoxin A in Roasted Coffee
Immunoaffinity Column HPLC Method
First Action 2000

(Applicable to the determination of ochratoxin A in coffee at > 1.2 ng/g.)

See Table **2000.09A** for the results of the interlaboratory study supporting acceptance of the method.

Caution: Ochratoxin A is a potent nephrotoxin and liver toxin that has been reported to have immunosuppressant properties. Prepare standards involving toluene in a fume hood. Keep containers with this solvent closed.

A. Principle

Ochratoxin A is extracted from roasted coffee by blending with methanol and sodium bicarbonate. The analyte is cleaned up by first passing through a phenyl silane column and then through an immunoaffinity column. Ochratoxin A is separated and identified by reversed-phase LC and quantified by fluorescence.

B. Apparatus

(**a**) *Silanized glass vials*.—To ensure stability of ochratoxin A in aqueous solvents, prepare vials by filling them with silanizing reagent, **C**(**m**). Wait 1 min, rinse vial twice with acetonitrile and twice with water, and dry.

(**b**) *Wrist action shaker*.

(**c**) *Refrigerated centrifuge*.—Operating at 4500 × *g* and 4°C.

(**d**) *Vacuum manifold*.—To accommodate phenyl silane columns and immunoaffinity column.

(**e**) *Vacuum pump*.—To pull a vacuum of 1000 Pa at 18 L/min for use with manifold, (**d**).

(**f**) *Filter papers*.—Whatman No. 4, or equivalent.

(**g**) *Phenyl silane solid-phase extraction columns*.—500 mg (e.g., J.T. Baker 7095-03, or equivalent).

(**h**) *LC apparatus*.—With the following: (*1*) *Valve injection system*.—With 100 µL injection loop. (*2*) *Mobile phase pump* (*isocratic*).—Pumping 1 mL/min with no detectable pulsation. (*3*) *LC column*.—C18, 5 µm particle size with 11% carbon loading, fully end-capped (pore size 100 Å), plus a corresponding reversed-phase guard column (2.5 cm × 4.6 mm id, e.g., Anachem C752 is suitable). (*4*) *Fluorescence detector*.—Emission wavelength 460 nm and excitation wavelength 333 nm. (*5*) *Data collection system*. (*6*) *Column oven*.—Set at 45 ± 1°C.

(**i**) *Spectrophotometer*.—For checking concentration of calibration solution.

(**j**) *Immunoaffinity columns*.—Specific for ochratoxin A cleanup. For example, columns from Vicam, LP (313 Pleasant St, Watertown, MA 02472) and Rhône Diagnostics (West of Scotland Science Park, Unit 306, Kelvins Campus, Glasgow G20 0SP, United Kingdom) meet these criteria. *Performance standards for immunoaffinity column*.—The immunoaffinity column should contain antibodies raised against ochratoxin A. The column should have a maximum capacity of not <100 ng ochratoxin A and should recover not <85% ochratoxin A when applied as a standard solution in CH_3OH–phosphate-buffered saline containing 5 ng of toxin.

(**k**) *Disposable syringe filters*.—0.2 µm pore size, 25 mm polysulfone membrane.

C. Reagents

(**a**) *Ochratoxin A stock standard solution*.—Prepare ca 10 µg/mL ochratoxin A standard solution in toluene–acetic acid (99 + 1). Determine absorbance of the 3 solutions of potassium dichromate, $K_2Cr_2O_7$, in 0.009M H_2SO_4 (0.25, 0.125, and 0.0625mM), at maximum absorption near 350 nm, against 0.009M H_2SO_4 as solvent blank. Calculate molar absorptivity (ε) at each concentration: ε = (A × 1000)/concentration in mM. If the 3 values vary by more than guaranteed accuracy of A scale, check either technique or instrument. Average the 3 ε values to obtain ε. Determine correction factor (CF) for particular instrument and cells by substituting in equation: CF = 3160/ε, where 3160 is the value for ε of $K_2Cr_2O_7$ solutions. If CF is <0.95 or >1.05, check either technique or instrument to determine and eliminate cause. (Use same set of cells in calibration and determination of purity.) The molar absorptivity of ochratoxin A should be taken as 5440 m^2/mol.

(**b**) *Methanol*.—HPLC grade.

(**c**) *Sodium bicarbonate*.—3 ± 0.5%. Dissolve 30 g $NaHCO_3$ and dilute with water to 1 L in volumetric flask.

(**d**) *Extraction solvent*.—Mix one part per volume methanol with one part per volume of the 3% $NaHCO_3$ solution, (**c**).

(**e**) *Phenyl silane column wash reagent 1*.—Mix methanol + the 3% $NaHCO_3$ solution (25 + 75; v/v).

(**f**) *Phenyl silane column wash reagent 2*.—1% $NaHCO_3$ solution. Dissolve 1 g $NaHCO_3$ in 100 mL water.

(**g**) *Phenyl silane column elution reagent*.—Mix methanol + water (7 + 93; v/v).

Table 2000.09A. Interlaboratory study results for ochratoxin A in roasted coffee

Matrix	No. of acceptable results	Mean value found for ochratoxin A, ng/g	Recovery, %	RSD_r, %	RSD_R, %	HORRAT
Blank (a)	13	0.1	—	27	71	1.2
NC[a] (b)	14	1.2	—	22	26	0.6
NC (c)	15	2.6	—	11	15	0.4
NC (d)	12	5.4	—	2	14	0.4
Spiked (4.0)	13	3.5	85	6	13	0.3

[a] NC = naturally contaminated.

(**h**) *Phosphate-buffered saline (PBS)*.—Dissolve 8 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, and 0.2 g KCl in 1 L distilled water. Adjust pH to 7.4 with 0.2M NaOH.

(**i**) *Acetonitrile*.—LC grade.

(**j**) *LC mobile phase*.—Mix water + acetonitrile + acetic acid (102 + 96 + 2, v/v/v).

(**k**) *Toluene–acetic acid mixture*.—Mix toluene + acetic acid (99 + 1, v/v).

(**l**) *Injection solution.*—Mix methanol + H_2O + acetic acid (30 + 70 + 1, v/v/v).

(**m**) *Silanizing reagent*.—SurfaSil-Pierce 428 is suitable.

D. Extraction

Weigh 15 ± 0.1 g test portion of roasted coffee into 500 mL conical flask. Add 150 mL extraction solvent, **C**(**d**). Stopper flask and shake gently for 30 min. Filter extract through filter paper, **B**(**f**). Transfer ca 50 mL portion of filtrate into centrifuge tube and centrifuge for 15 min at 4°C at 4500 × *g*.

E. Phenyl Silane Column Cleanup

Attach phenyl silane column, **B**(**g**), to vacuum manifold, **B**(**d**). Prepare column, **B**(**g**), with 15 mL methanol, **C**(**b**), followed by 5 mL 3% $NaHCO_3$, **C**(**c**). Do not let column run dry. Discard washings. Pipet 10 mL centrifuged coffee filtrate into glass beaker and add 10 mL extraction solvent, **C**(**d**). Pass this diluted extract through the column, at maximum speed of 5 mL/min. Wash column, with 10 mL wash reagent 1, **C**(**e**), followed by 5 mL wash reagent 2, **C**(**f**). Remove column from vacuum manifold and place over collection vessel. Dry the column with ≥three 10 mL volumes of air using a syringe. Elute ochratoxin A with 10 mL column elution reagent, **C**(**g**).

F. Immunoaffinity Column Cleanup

Dilute the eluate with 30 mL PBS, **C**(**h**). Connect immunoaffinity column, **B**(**j**), to vacuum manifold, **B**(**d**), and attach a reservoir to the immunoaffinity column. Add diluted test extract to reservoir and pass through immunoaffinity column. Flow rate should not exceed 5 mL/min. Do not permit column to run dry. Wash column with 10 mL water and dry by applying vacuum for 5–10 s or passing air by syringe for 10 s. Remove column from vacuum manifold and place over a silanized vial, **B**(**a**). Elute ochratoxin A into silanized vial, **B**(**a**), with four 1 mL portions of methanol, **C**(**b**). Evaporate eluate to dryness over steam bath, under N. Redissolve in 1 mL injection solution, **C**(**l**), which has been filtered through 0.2 μm filter, **B**(**k**), and transfer to LC vial.

Table 2000.09B. Preparation of calibrant standard solutions

Calibrant solution	Concn ochratoxin A, ng/mL	Ochratoxin A working standard solution, mL	Injection solution, mL
1	0.5	125	4875
2	1.0	250	4750
3	2.0	500	4500
4	5.0	1250	3750
5	10.0	2500	2500

G. LC Determination

(**a**) *Calibration graph*.—Prepare a calibration graph at the beginning of each set of analyses and whenever the chromatographic conditions change.

(**b**) *Preparation of intermediate standard solution*.—2 μg/mL. Pipet 200 μL ochratoxin A stock standard solution, **C**(**a**), into silanized glass vial and add 800 μL toluene– acetic acid mixture (99 + 1), **C**(**k**). Store solution at –18°C for <4 weeks.

(**c**) *Preparation of working standard solution*.—20 ng/mL. Pipet 100 μL of solution, (**b**), into a silanized glass vial, **B**(**a**), and evaporate under a stream of N. Redissolve in 10 mL methanol, **C**(**b**), that has been filtered through a 0.2 μm filter, **B**(**k**). Use this solution to prepare calibrant solutions 1–5 as in Table **2000.09B**, using filtered injection solution, **C**(**l**), for dilution.

(**d**) *Operating conditions*.—When the column specified in **B**(**h**) and the mobile phase specified in **B**(**j**) were used, the following settings were found to be appropriate: flow rate, 1 mL/min; column oven temperature, 35 ± 1°C; fluorescence detection, emission wavelength 460 nm, excitation wavelength 333 nm; injection volume, 100 μL.

H. Calculation

Determine from the calibration graph, the masses of ochratoxin A in the aliquot of test solution injected onto the LC column. Calculate the mass fraction, W_{OTA}, of ochratoxin A in μg/kg as follows:

$$W_{OTA} = M_A \frac{V_1}{V_2} \frac{V_3}{V_4} \frac{1000}{M_S} \frac{1}{1000}$$

where M_A = mass of ochratoxin A in aliquot of test solution injected on column, ng; V_1 = volume of extraction solvent, mL (150 mL); V_2 = volume of test filtrate taken for cleanup, mL (10 mL); V_3 = volume of test solution, mL (1 mL); V_4 = volume of aliquot of test solution injected on column, mL (0.1 mL); M_S = mass of test portion extracted, g (15 g); 1000 = factor to convert g to kg; $^1/_{1000}$ = factor to convert ng to μg.

Reference: *J. AOAC Int*. **84**, 444(2001).