AOAC Official Method 994.08
Aflatoxins in Corn, Almonds, Brazil Nuts, Peanuts, and Pistachio Nuts

Multifunctional Column (Mycosep) Method
First Action 1994
Final Action 1997

(Applicable to determination of 5–30 ng total aflatoxins/g in corn, almonds, brazil nuts, peanuts, and pistachio nuts.)

See Table **994.08A** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Test portion is extracted with acetonitrile–H_2O solution (9 + 1). Extract is filtered and then applied to multifunctional column containing mixture of reversed-phase, ion exclusion, and ion exchange adsorbents. Packing retains interferences such as fats, proteinaceous compounds, pigments, and carbohydrates extracted from food and feed ingredients. Aflatoxins are eluted from column, derivatized with trifluoroacetic acid, and quantitated by liquid chromatography with fluorescence detection.

B. Apparatus

(**a**) *Cleanup column*.—With lipophilic and charge active sites packing housed in 6 mL plastic tube (1.0 × 10 cm) with rubber flange on lower end; porous frit in center of flange; 1-way valve above frit (Mycosep™ 224 MFC Column, Romer Labs, Inc., Washington, MO 63090, USA, or equivalent). Store ≤1 year at room temperature. To check column performance pass through column 0.5 mL acetonitrile–H_2O solution (9 + 1) containing 10 ng total aflatoxins B_1, B_2, G_1, and G_2/mL (5:1:3:1). Recovery of each aflatoxin should be >90%.

(**b**) *LC injection system*.—Calibrated to deliver 50 µL.

(**c**) *Liquid chromatography (LC) pump*.—Capable of delivering 2.0 mL/min.

(**d**) *LC column*.—4.6 mm × 10 cm, 5 µm, C_{18} (Brownlee LC cartridge 0711–0015 and cartridge holder 0715–0014, or equivalent), flow rate 2.0 mL/min; or 3.9 mm × 15 cm, 5 µm, C_{18} (Waters, µBondaPak 086684, or equivalent), flow rate 1.0 mL/min. A 25 cm column can be used with mobile phase components at appropriate concentrations. Mobile phase composition and LC column may vary as long as peaks are baseline resolved for all 4 aflatoxins, response is linear with concentration, response and chromatograms are reproducible, and quantitation of known test sample is accurate.

(**e**) *Fluorescence detector*.—Operating conditions (nm): excitation 360, emission 440. Shimadzu Model RF-535 is suitable.

(**f**) *Blender/shaker*.—With 250 mL blender jar and cover; rotary or wrist action shaker.

(**g**) *Filter paper*.—25.5 cm, qualitative.

(**h**) *Pipets*.—With tips, capable of delivering 3 mL.

(**i**) *Culture tube*.—15 × 85 mm, borosilicate.

(**j**) *Syringe*.—1000 µL, graduated, glass.

(**k**) *Derivatization vial*.—2.0 mL, with Teflon-lined cap.

Table 994.08A Interlaboratory study results for determination of aflatoxins

Commodity	Total aflatoxins added, ng/g	s_r	RSD_r, %	s_R	RSD_R, %	Rec., %
Corn	5			1.2	22.8	102
	10			1.8	17.7	100
	20	1.2	6.0	4.4	22.3	98
	30			4.8	15.4	105
	NC[a]	4.3	20.4	4.3	20.4	—
Almonds	5	0.7	16.4	1.2	26.3	90
	10			1.8	19.6	94
	20			4.1	22.0	93
	30			5.0	17.2	96
Brazil nuts	5			2.1	29.5	140 (70)[b]
	10			1.4	12.0	113 (78)[b]
	20			3.4	17.0	101 (84)[b]
	30	2.5	8.6	3.6	12.4	97 (85)[b]
Peanuts	5			2.6	69.4	74
	10	0.6	6.8	2.1	25.0	86
	20			4.8	26.2	91
	30			5.4	21.7	83
Pistachios	5			1.7	33.6	92
	10			2.5	27.4	92
	20	4.2	23.2	5.8	31.8	91
	30			5.5	19.8	92

[a] Naturally contaminated corn at 23 ng total aflatoxins/g.

[b] Recoveries corrected for background levels.

C. Reagents

(**a**) *Extraction solvent*.—Mix 900 mL acetonitrile (reagent grade) with 100 mL H_2O.

(**b**) *Derivatization solution*.—Mix 10 mL trifluoroacetic acid (reagent grade) with 5 mL glacial acetic acid (reagent grade) and 35 mL H_2O. This volume is sufficient for 70 derivatizations.

(**c**) *LC mobile phase*.—(*1*) *10 cm C_{18} column*.—Mix 100 mL acetonitrile (LC grade) with 400 mL H_2O. (*2*) *15 cm C_{18} column*.—Mix 100 mL acetonitrile with 100 mL methanol, and 400 mL H_2O. De-gas, and store in capped container when in use. Adjust composition of mobile phase so retention times of 4 aflatoxins are 4–11 min.

(**d**) *Standard stock solution*.—Dilute mixed aflatoxin stock solution in benzene–acetonitrile (98 + 2) as in **971.22B**–**E** (*see* 49.2.03) to contain 300 ng B_1, 50 ng B_2, 150 ng G_1, and 50 ng G_2/mL. Refrigerate when not in use.

(**e**) *Standard working solutions*.—Transfer aliquot of standard stock solution, (**d**), (Table **994.08B**) into 10 mL volumetric flask. Evaporate just to dryness under stream of nitrogen at room temperature. Dilute residue to mark with acetonitrile.

D. Extraction

Weigh 50 g test portion into blender jar or 250 mL Erlenmeyer flask. Add 100 mL extraction solvent, **C**(**a**). Blend 2 min or shake at high speed 1.0 h. Filter and collect extract. Pipet 3 mL extract into 10 mL culture tube.

E. Multifunctional Column Chromatography

Hold cleanup column in one hand, and glass culture tube containing 3 mL extract in other. Slowly push cleanup column (rubber flange end) into culture tube. Rubber flange creates tight seal with glass wall of culture tube. As column is pushed further into tube, extract is forced through frit, through 1-way valve, and through packing material. Collect ca 0.5 mL purified extract in column reservoir. (*Note*: Do not place finger over top of cleanup column reservoir.) Quantitatively transfer 200 μL purified extract from top of column to derivatization vial.

F. Aflatoxin Derivatization

Place 200 μL working standard solution, **C**(**e**), or 200 μL purified extract from **E**, into derivatization vial, and then add 700 μL derivatizing solution using 1000 μL syringe. Close vial with cap and mix solution well. Heat vial ≥8.5 min (*Note*: That time is necessary for complete derivatization of aflatoxin B_1 or G_1.) in 65°C water bath (level of water in bath must be above level of solution in vial.) (*Caution*: Cool to room temperature before opening the vial.) Proceed with LC.

Table 994.08B Preparation of standard working solutions

Standard working solution	Stock solution, μL/10 mL	Derivatized aflatoxins mixture in standard working solution, ng/50 μL			
		B_1	B_2	G_1	G_2
1	270	0.090	0.015	0.045	0.015
2	180	0.060	0.010	0.030	0.010
3	90	0.030	0.005	0.015	0.005

G. Liquid Chromatography

Run entire system 10–20 min to stabilize it. When using integrator, adjust sensitivity controls of fluorescence detector to give reasonable integrator response (signal:noise = 5:1) for lowest concentration of standard working solution (Table **994.08B**, solution 3). If strip chart recorder is used, adjust controls to give 5% deflection for smallest peak. Inject 50 μL derivatized standard working solution from **F**. Aflatoxins elute in order: G_{2a}, B_{2a}, G_2, B_2. When using 10 cm column, retention times of 4 aflatoxins are ca 2, 2.8, 6, and 8 min, respectively. When using 15 cm column, retention times of 4 aflatoxins are ca 6, 8, 9, and 11 min, respectively. Inject 50 μL derivatized standard working solutions (Table **994.08B**) and prepare standard curve for each aflatoxin.

Plot response vs quantity (ng aflatoxin/50 μL derivatized mixture). Inject 50 μL derivatized extract from **F**. Identify each aflatoxin peak in derivatized extract chromatogram by comparing its retention time with corresponding peak in standard chromatogram. Determine quantity of each aflatoxin, *C*, in derivatized extract (injected) from respective standard curves.

If using strip chart recorder and peak responses for derivatized extract are off scale, dilute extract with mobile phase and reanalyze it to bring peaks on scale. Calculate concentration of each aflatoxin in test sample as follows:

$$W = 50\ \text{g} \times (0.2\ \text{mL}/100\ \text{mL}) \times (0.05\ \text{mL}/0.90\ \text{mL}) = 0.00555\ \text{g (no dilutions)}$$

$$\text{aflatoxin ng/g (ppb)} = C/W$$

where W = equivalent weight of test portion injected (in 50 μL) into LC, adjusted for dilution if necessary; C = ng aflatoxin (in 50 μL) injected into LC.

Reference: *J. AOAC Int.* **77**, 1512(1994).

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