AFLATOXINS (Quantitative Procedure)

PRINCIPLE

Aflatoxins are extracted with aqueous methanol. Interfering substances are removed by affinity chromatography. Retained pure aflatoxins are eluted from affinity column with methanol. An estimate of total aflatoxins is made by derivatization with bromine followed by a fluorometric measurement. For quantitative analysis, the affinity column eluent is separated by revised phase chromatography followed by derivatization with iodine. The individual derivatives are detected by fluorometric detector and quantitated relative to standard by computing integrator.

SCOPE

This method is applicable to ground corn. With appropriate modification, it may also be applied to other corn wet milling process samples, like starch, steepwater corn germ and corn germ meal, corn gluten meal, corn gluten feed and corn fiber. Sample clean-up procedure is also applicable to determinations of aflatoxins by thin layer chromatography.

CAUTION

Aflatoxins should be handled as very toxic substances. All manipulations of aflatoxins must be performed in a fume hood. Use hypochlorite bleach (diluted 1:5 with water) to treat spills and to destroy any aflatoxins which come in contact with glass or plastic ware.

SPECIAL APPARATUS

1. High Speed Blender: explosion proof, with 1-quart steel jar and lid, such as Waring Blender. Available from fisher Scientific, Catalog No. 14-509-19.

2. Borosilicate Glass Disposable Culture Tubes: 16 mm × 125 mm, Fisher Scientific, Catalog No 14-962-10E, or equivalent.

3. Pipettes: 5000, 1000, 500, 200, 100 µL capacity, Eppendorf, or equivalent.

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4. Polypropylene Round Bottom Tubes: 17 mm × 100 mm, Fisher Scientific Catalog No. 14-959-12A, or equivalent.

5. Vortex Mixer: Fisher Scientific, Catalog No. 2-814-5, or equivalent.

6. Funnels: 75 mm × 150 mm stem, Fisher Scientific Catalog No. 10-326-C, or equivalent.

7. Filter Paper: 18.5 cm, Whatman #1, Fisher Scientific Catalog No. 09-805-H, or equivalent.

8. Aflatest-P Aflatoxin Affinity Columns: Including 10 mL glass syringe and syringe pump, available from VICAM, 29 Mystic Avenue, Somerville, MA 02145.

9. Nylon 25 mm Acrodisc 0.45 μm Membrane Filter: Product No. 4438, Gelman Sciences, available from Fisher Scientific.

10. High Performance Liquid Chromatograph: Equipped with post column reactor and fluorescence detector (Figure 1), as follows:

    A. Pulseless Pumps: Two Waters Model 5110, Waters Associates, Milford, MA 01757, or equivalent.

    B. Injector: Waters 812 WISP autosampler or Waters U6K loop injector, Waters Associates, or equivalent. Equivalent injectors must be capable of handling small (200 μL) and variable sample volumes.

    C. Analytical Column: Packed with Partisil 5 ODS-3 silica, Whatman Catalog No. 4238-001, or equivalent.

    D. Precolumn: 3 cm Brownlee RP-18 cartridge and cartridge holder, or equivalent. Available from Rainin Instrument Co., Inc., Woburn, MA 01801, Catalog Nos. OD-GU and 140-20.

    E. Post Column Reactor: Kratos PCRS 520, from ABI Analytical, Kratos Division, Ramsey, NJ 07446, or equivalent.

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F. Detector: Spectro Vision FD-100 filter fluorometer with 10 W xenon flash lamp, 365 nm excitation filter, from Spectro Vision, Inc., 25 Industrial Avenue, Chelmsford, MA 01824, or equivalent.

G. Computing Integrator: Shimadzu Chromatopac C-R6A, from Shimadzu Scientific Instruments, Inc., Columbia, MD 21046, or equivalent.

11. Fluorometer: Sequoia-Turner Model 450 or equivalent.

12. Standards for Fluorometer: Available from VICAM.

REAGENTS

1. Purified Water: Filter and repurify deionized water by reverse osmosis, e.g., by means of a Milli-Q system. Available from Millipore Co., Bedford, MA 01730.

2. Methanol: Reagent grade distilled in glass.

3. Aflatoxin Standards: Mixed, containing 5 µg/,L each of aflatoxin B_1 and $G_1$and 1.5 µg/mL each of aflatoxin B_2 and G_2 in benzene: acetonitrile (98:2) from Applied Science Laboratories, Inc., P.O. Box 440, State College, PA 16801, Catalog No. 17621. Keep frozen before use. If reassay is desired, follow the procedure in the Official Methods of Analysis of the AOAC, 15th Edition (1990), 971.22, pp. 1186-1187.

4. Filter Aid: Celite, acid washed, or equivalent.

5. Nitrogen Gas: Dry, reagent grade.

6. HPLC Elution Solvent, 50% Aqueous Methanol, v/v: Add equal volumes of reagent grade methanol and purified water and mix well; degas by stirring under vacuum for a few minutes.

7. Post-column Derivatization Reagent, 0.01% Aqueous Iodine, w/v: Dissolve 0.100 g iodine in 1 mL methanol. Transfer quantitatively with the

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aid of little methanol to a 1 L volumetric flask containing about 800 mL degassed purified water. Stir until solution is homogeneous and clear. Dilute to mark with purified water and mix. Prepare fresh daily.

8. Methanol: HPLC grade.

9. Developing Solution (x% Bromine): Available from VICAM.

PROCEDURE

Sample Preparation: From a thoroughly blended, representative laboratory sample (Note 1), passing through 20 mesh screen (Note 2), weigh 50 g ground corn. Transfer this analytical sample into a 1 quart blender jar, add 5 g Celit filter aid, 5 g sodium chloride, 200 mL 85% methanol in water; blend at high speed for 3 mins. (Notes 3). Filter through 18.5 cm Whatman #1 paper and collect 15 mL filtrate in a 16 mm × 125 mm glass test tube.

Affinity Chromatography Over Aflatest-P Column: Take a 5 mL aliquot of the filtrate filter through a 25 mm nylon Acrodisc 0.45 µm pore size membrane filter. Attach a 10 mL syringe barrel to an Aflatest-P™ bound antibody column by means of an adapter. Transfer the 5 mL clear solution to the syringe barrel quantitatively, with the aid of little 10% aqueous methanol. Remove the column end cap, depress the syringe pump and push the solution through the antibody column into a waste receptacle at a rate of 2 mL/min. or in about 3 mins. Disconnect the syringe pump, add 10 mL purified water to the barrel, reattach the pump, push the water through the column into waste; repeat and follow this second rinse by pushing a small volume of air to remove excess water. Place a small glass test tube under the column outlet, pipet 1.0 mL HPLC grade methanol into the syringe, push through quickly to elute the aflatoxins. Swirl the collected solution in a Vortex mixer at low speed. This solution can now be analyzed wither (1) fluorometrically or (2) chromatographically.

Detection of Aflatoxins Using a Fluorometer: Add 1 mL of fresh developing solution to the 1 mL of eluent from the Aflates-P column. Mix well and measure in a calibrated fluorometer. The fluorometer is standardized by using the 0 and 20 ppb standards.

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Standardization of Fluorometer: Different fluorometers can use different values for the calibration. The recommended settings for Sequoia-Turner Model 450 are:

0 = !3
20 = 100

Liquid Chromatography and Post Column Derivatization: The system consists of an autosampler or a loop injector, two pulseless pumps (one for the eluent and one for the iodine reagent solution), a column heater cabinet also holding a post column mixing-reaction coil, fluorescence detector and a computing-recording integrator. The arrangement of these components is shown in Figure 1. Typical analytical parameter are shown below:

Chromatographic Column:	Whatman Partisil 5 ODS-3 or equivalent
Column Temperature:	40 °C
Eluent	Methanol: Water (1:1, by volume)
Eluent Flow Rate:	0.7 mL/min.
Post Column Reagent:	0.1 g/L I_2 in water; prepare fresh daily
Reagent Flow Rate:	0.4 mL/min.
Reaction Coil Temperature:	75 °C
Detector Excitation Wavelength:	365 nm
Detector Emission Filter Wavelength:	400 nm
Detector Sensitivity:	10
Detector Response:	1
Detector Lamp Frequency:	30 – 1 ns
Injection Volume:	25 µL

Preparation of Standards: Mixed standards containing 5 µg/mL each of aflatoxins B_1 and G_1, and 1.5 µg each of aflatoxins B_2 and G_2 in benzene:acetonitrile 98.2, are recommended. These are kept frozen. Make a 10-fold dilution of the contents of one ampoule (5 mL) in a 50 mL volumetric flask with benzene:acetonitrile, 98:2; distribute 1 mL aliquots in amber vials with Teflon lined caps, seal tightly and refreeze. Just before use, thaw one vial and take 0.5 mL aliquots, evaporate to dryness (Note 4) under a stream of nitrogen in a polypropylene test tube and redissolve in 80% aqueous methanol.

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Run standard solution at 50 ppb aflatoxins B_1 and G_1, and 15 ppb B_2 and G_2 in 80 % aqueous methanol (Note 5), at least, every 20 consecutive injections.

CALCULATION, Fluorometer

Using recommended settings:

$$\text{Aflatoxins} = \frac{\text{reading}}{5} \times 2.5 = \frac{\text{reading}}{2}$$

CALCULATIONS, HPLC (Note 6)

$$\text{Aflatoxin } B_1 \text{ or } G_1\text{, ppb} = \frac{\text{Peak Area of Sample}}{\text{Peak Area of Standard}} \times 100$$

$$\text{Aflatoxins } B_1 \text{ or } G_1\text{, ppb} = \frac{\text{Peak Area of Sample}}{\text{Peak Area of Standard}} \times 30$$

NOTES AND PRECAUTIONS

1. A “laboratory sample” is material as received by the laboratory. A subsample taken for analysis is an “analytical sample”.

2. Whole corn should be ground through 10 mesh or less and thoroughly blended before an analytical sample is taken. AOAC method calls for 20 mesh. See AOAC Official Methods of Analysis, 15th Edition (1990), 99.16, p. 1185.

3. A 500 mL stoppered Erlenmeyer flask placed on a mechanical shaker may be used for extraction; shake at high speed for 30 mins.

4. Use of glass to distill/evaporate aflatoxin solutions to dryness causes losses due to binding of the compound(s) to the silicate surface of the container.

5. For other information on availability and handling of aflatoxin standards, see Official Methods of Analysis of the AOAC, 16th Edition (1995), Vol. II, Ch. 49, p. 3, Method 970.44, “Preparation of Standards for Mycotoxins”.

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6. Actual calculations to obtain parts per billion of aflatoxins with respect to the original 50 g test sample extracted are as follows:

$$\text{Aflatoxin, ppb} = \frac{(\text{Peak Area}_{Spl})(C_{Std}, \mu g/mL)(V_{Std}, \mu L)(D_{Spl}, \mu L)}{(\text{Peak Area}_{Std})(V_{Spl}, \mu L)(W_{Spl}, g)}$$

where:

(Peak Area $_{spl}$)	=	Peak Area of Sample
(Peak Area $_{Std}$)	=	Peak Area of Standard
C_{Std}	=	Concentration of Standard, Typically, 0.05 µg/mL for B_1 and G_1, and 0.015 µg/mL for B_2 and G_2
V_{Std}	=	Volume of injected standard
D_{Std}	=	Volume of methanol used to elute Aflatoxins from affinity column
V_{Spl}	=	Volume of sample solution Injected
W_{Spl}	=	Weight of sample extracted and Carried through clean-up or (50 g/200 mL) × 2.0 mL = 0.50 g for this method.

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