Method 24.0

Aflatoxin Determination

Purpose: To determine the aflatoxins B_1, B_2, G_1, G_2, in ginger and other spices unless specified. Procedure not applicable to Black Pepper.

A. Apparatus:

1. Shaker--Burrell wrist action.

2. Chromatographic column--22x300 mm. reservoir type (250 mL capacity) with Teflon stopcock.

3. TLC plates--20x20 cm. uniplates precoated with Silica GHR 250 microns thick. Activate 1 hr. at 110°C. and store in desiccator. (Analtech, Inc., Blue Hen Industrial Park, South Chapel St. Ext., Wilmington, DE 19811).

4. Micro-pipets "Microcaps"--3 µl. & 4 µl. capacity (Biolab, Inc., 359 Main Street, Reading, MA 01867).

5. Chromato-Vue cabinet--(Ultra Violet Products, Inc. 5114 Walnut Grove Ave., San Gabriel, CA 91778) or equivalent.

6. Funnel--150 mm. diameter with fluted No. 1 Whatman filter paper (32 cm. diameter).

7. Graduated cylinders--10 mL and 250 ml.

8. Separatory funnel--250 mL capacity.

9. Vials--4 dram capacity (14.8 mL).

10. Developing tank.

11. Plastic wrap--Saran or equivalent.

12. Hot water bath.

B. Reagents:

1. Silica Gel 60--Brinkmann #7734, 70-230 mesh (Brinkmann Instruments, Inc., Cantiague Road, Westbury, NY 11590). Dry 2 hours at 110°C.

2. Sodium sulfate-anhydrous, granular

Method 24.0

Aflatoxin Determination

3. Aflatoxin standard--No. 4-6300 (Supelco, Inc., Supelco Park, Bellefonte, PA 16823). B_1, and G_1, 1μg/mL; B_2, and G_2, 0.3 μg/mL.

4. Dilute HCI (0.1 N.)--dilute 0.89 mL conc. HCI to 100 mL H_2O.

5. Lead acetate solution---dissolve 50 g neutral lead acetate trihydrate in distilled H_2O, add 3 mL glacial acetic acid, and dilute to 1 liter.

6. Sulfuric acid--25% aqueous solution.

7. Celite 545.

8. Carborundum #12 granules.

9. Solvents--reagent grade:
   a. Acetic acid--glacial.
   b. Acetone.
   c. Toluene.
   d. Methanol.
   e. Ether--anhydrous.
   f. Hexane.
   g. Methylene chloride.
   h. Chloroform.
   i. Acetonitrile.

10. Toluene: acetic acid (3+1)--150 mL toluene + 50 mL acetic acid, made in 250 mL graduated cylinder.

11. Ether: hexane (3+1) 150 mL anhydrous ether + 50 mL hexane, made in 250 mL graduated cylinder.

12. Methylene chloride: acetone (3+1)--150 mL methylene chloride + 50 mL acetone, made in 250 mL graduated cylinder.

13. Toluene: acetonitrile (8+2)--8 mL toluene + 2 mL acetonitrile, made in 10 mL graduated cylinder.

14. Developing solvents:
    1st dimension solvent--10% acetone in chloroform.
    2nd dimension solvent--toluene 55%, chloroform 29.5%, acetic acid 15%, water 0.5%.

Method 24.0

Aflatoxin Determination

C. Preparation of Sample:

1. Grind 200 g of whole samples in Waring blender (high speed) prior to extracting.

2. For analysis of oleoresins, dissolve 2.0 g of sample in 10 mL of methylene chloride and proceed with the column chromatography. (D.5)

D. Procedure: (Note 1)

1. Weigh 50 g sample into 500 mL Erlenmeyer flask, add 70 mL of 0.1 N. HCI, and shake to wet entire sample.

2. Add 10 g Celite 545 and 250 mL methylene chloride. Stopper with an aluminum foil covered stopper. Shake 30 min. on shaker.

3. Filter through fluted filter paper and collect 100 mL of filtrate.

4. Evaporate to *near* dryness on hot water bath and dissolve in 10 mL methylene chloride. (Note 2)

5. Prepare column for column chromatography:
   a. Place ball of glass wool in bottom of chromatographic tube and tamp gently (ca. 5 mm).
   b. Fill column about 1/3 full of methylene chloride and pour in 5 g sodium sulfate.
   c. Slurry 15 g silica gel in 100 mL beaker with 40 mL methylene chloride and pour into column. Wash sides with small amount of methylene chloride.
   d. When gel settles, top carefully with 5 g sodium sulfate and drain column to just above sodium sulfate layer.

6. Transfer the filtrate to the column, washing the beaker with 5 mL methylene chloride. Allow solution to just pass through the top of the sodium sulfate.

7. Eluting at maximum flow, wash column with 200 mL of toluene: acetic acid solution, followed by 200 mL of ether: hexane solution. Discard both washes.

8. Elute aflatoxins with 200 mL of methylene chloride: acetone solution; collect this fraction from time of addition until flow stops.

9. Add a few glass beads to eluate and evaporate to *near* dryness (ca. 1 mL) on hot water bath.

Method 24.0

Aflatoxin Determination

10. Dissolve residue in 20 mL acetone.

11. While stirring, add 80 mL lead acetate solution. Let stand for a few minutes.

12. Add 5 g Celite 545 and filter through Whatman #1 filter paper.

13. Place 50 mL of filtrate in 250 mL separatory funnel. Extract the aflatoxins twice with 50 mL portions of methylene chloride. (Note 3)

14. Combine both methylene chloride extracts (lower layer), add a few glass beads, and evaporate to *near* dryness on hot water bath.

15. Transfer residue quantitatively to a 4 dram vial with methylene chloride, add carborundum #12 granules, and evaporate to dryness under gently stream of nitrogen.

16. Immediately prepare thin layer developing solvents and add 80 mL of each solvent to separate tanks. (Note 4)

17. Gently insert a glass trough into the bottom of each tank; add approximately 25 mL of water to each. Cover and see #20. Allow tanks to equilibrate for ½ hour.

18. Dissolve sample extract in 0.5 mL toluene: acetonitrile and mix thoroughly on Vortex mixer.

19. In subdued incandescent light, spot plates.

20. Develop plate in 1st dimension for ca. 12 cm. of solvent travel; be sure tank is sealed with plastic wrap and covered to exclude light.

21. Remove plate and air dry in subdued light.

22. Look at TLC plates under long-wave UV light noting color differences. Bluish fluorescence of "B" contrasted with Greenish "G" aflatoxins. The aflatoxins are in order of decreasing R_f,: B_1, B_2, G_1, G_2.

23. Develop sample streak in second dimension for about 12 cm. of solvent travel using second developing solvent. Allow to air dry as done for first dimension.

24. View plate under long-wave UV light in Chromato-Vue cabinet.

25. Compare sample with standard, observing pattern of four fluorescent spots.
    a. In order of decreasing R_f,: they are B_1, B_2, G_1, and G_2.
    b. Note small color differences.

26. Spray plates with 25% aqueous solution of sulfuric acid and observe under long-wave UV light.
    a. Aflatoxins B_1 and B_2 are bright yellow, while G_1 and G_2 are blue-yellow.
    b. Color change is indicative of, but not conclusive evidence of, aflatoxins; however, failure of suspect spots to exhibit color change rules out presumptive aflatoxins. (Note 5&6)

E. Calculations:

The sample chromatogram is matched with the R_f values and color intensity of the standards.

$$\text{ppb of aflatoxin} = \frac{\text{Wt. of aflatoxin in the spot}}{\textbf{Wt. of sample on the plate}}$$

For example: If aflatoxin B_1 spot of the sample matches B_1 spot from a 6µl. aliquot of the standard, and the B_2 spot is one-half the intensity of the B_2 standard.

a 20µl. sample aliquot would represent:

$$50 \times \frac{100}{250} \times \frac{50}{100} \times \frac{0.02}{0.50} = 0.4 \text{ g original sample}$$

Wt. of aflatoxin B_1 in the 6µl. aliquot of the standard:

$$B_1 = \frac{1.0 \times 10^{-6}}{1 \text{ mL.}} \times 6 \times 10^{-3}\text{ml.} = 6.0 \times 10^{-9}\text{g.}$$

$$\text{Ppb. } B_1 \text{ in sample} = \frac{6.0 \times 10^{-9}}{0.4} = 15 \times 10^{-9} \text{ or 15 ppb. in the sample}$$

Wt. of aflatoxin B_2 in the 6 µl. aliquot of the standard:

$$B_2 = \frac{0.3 \times 10^{-6}\text{g}}{\text{ml}} \times 6 \times 10^{-3}\text{mL} \times 0.5 = 0.90 \times 10^{-9}\text{g.}$$

Method 24.0

Aflatoxin Determination

Ppb. B_2 in sample = $\frac{0.90 \times 10^{-9}\text{g}}{0.4\text{ g}} = 2.25 \times 10^{-9}$ or 2.3 ppb. in the sample.

Total aflatoxin in sample: 15.0 + 2.3 = 17.3 ppb.

A similar calculation is performed for $G_1 + G_2$ if present.

F. Statistics:

TBD

G. Notes:

1. Procedure consists of four stages: Aflatoxin isolation and concentration (steps 1-4).
   Column preparation (steps 5-9).
   Lead acetate purification (steps 10-15).
   Thin layer chromatography (steps 16-26).

2. For storage overnight, evaporate to 1-2 mL, cap vial with aluminum-lined cap, cover entire vial with aluminum foil, and refrigerate. Mild heating during evaporation has not been found to affect results adversely.

3. Do not expose the extractions to light any longer than is absolutely necessary.

4. Presence of water vapor in TLC tank improves aflatoxin separations.

5. All trash from the analysis is to be disposed of in a properly labeled sealed plastic bag.

6. All glass is to be soaked in bleach to detoxify.

H. References:

AOAC Official Methods of Analysis (1995) 49.2.08 (968.22).