

CHAPTER 8

AFLATEST TEST METHOD

<u>Section Number</u>	<u>Section</u>	<u>Page Number</u>
8.1	GENERAL INFORMATION.....	8-1
8.2	PREPARATION OF SOLUTIONS	8-1
8.3	FLUOROMETER CALIBRATION	8-2
8.4	SOLUTION TESTING	8-5
8.5	TEST PROCEDURES	8-6
8.6	CLEANING LABWARE	8-15
8.7	WASTE DISPOSAL	8-15
8.8	EQUIPMENT AND SUPPLIES	8-16
8.9	STORAGE CONDITIONS	8-17

8.1 GENERAL INFORMATION

The Aflatest method of testing for aflatoxin uses monoclonal antibody affinity chromatography that provides for quantitative measurement of total aflatoxins (B1, B2, G1, and G2) in parts per billion (ppb) or qualitative (screening) for aflatoxin.

8.2 PREPARATION OF SOLUTIONS

Prior to beginning test procedures, prepare the solutions required for testing. The distilled/deionized water, dilute developer solution, and the HPLC grade methanol must be checked for background fluorescence with the fluorometer after properly calibrated. None of the above reagents should give a positive reading of more than 1.0 ppb.

a. Dilute Developer Solution.

NOTE: Developer Solution must be prepared fresh daily.

The concentrated developer solution should have a slight reddish brown color. (Do not use the stock solution if it is colorless.) Loss of color indicates that the stock solution has lost its potency.

Prepare dilute developer solution by adding 5 ml of Aflatest developer concentrate (Vicam Cat. # 32010) to 45 ml of distilled/ deionized water. Mix well, and label the dilute developer solution bottle showing the date and time of preparation.

DO NOT USE IT AFTER 6 HOURS HAVE ELAPSED.

If the amount of dilute developer being prepared needs to be adjusted based on the workload at individual locations, make sure that the 1 part concentrated developer to 9 parts distilled/deionized water ratio is maintained.

Label each stock bottle of concentrated developer with the date on which it was first opened. **DO NOT USE IT AFTER 30 DAYS HAVE ELAPSED.**

b. 80/20 Percent Methanol Solution.

Make up the solution by using the ratio of 8 parts HPLC grade methanol to 2 parts deionized/distilled water. Prepare the 80 percent methanol water solution by adding 800 ml methanol to 200 ml of water. Mix well. Keep the bottle tightly capped when not in use.

Label the 80 percent methanol/water solution bottle showing date of preparation. If the amount of the 80 percent methanol solution being prepared needs to be adjusted based on the workload at individual locations, make sure that the 8 parts HPLC grade methanol to 2 parts distilled/deionized water ratio is maintained.

To prepare smaller or larger amounts of solution the ratio of 8 parts methanol to 2 parts of deionized or distilled water must be maintained. For example: To prepare a solution that will provide for 5 test extractions (100 ml per test sample) mix 400 ml HPLC grade methanol to 100 ml deionized or distilled water.

8.3 FLUOROMETER CALIBRATION

a. General.

An FGIS-approved fluorometer is used to determine the aflatoxin level. To ensure accurate results, calibrate the fluorometer prior to use each day and verify at least once an hour using the **Yellow Vial**.

Turn the fluorometer on with the On/Off switch located on the rear panel. When the fluorometer is turned on, allow it to warm up for 10 minutes before calibrating. Once the fluorometer is turned on, it may be left on until close of business for the day. If the fluorometer is turned off during the day, a 10-minute warm up is required.

After turning the fluorometer on, it will identify itself and perform a set of self-tests. If any error message appears, consult the operator's manual.

b. Calibration Procedures.

- (1) Set the date, time, test delay time (60 seconds), and measurement units (ppb).
- (2) Follow the prompts on the fluorometer display to calibrate the unit.
- (3) When prompted to insert a calibration vial, wipe the vial with a clean cloth or paper wipe and insert it into the bottom of the well. Be sure that the vial is fully inserted and touches the bottom of the well.

- (4) Enter the correct calibration value (see table below) for the high calibrator (red vial) and low calibrator (green vial).

Note: This step is applicable to the Series III and Series IV fluorometers only. Calibration values are not entered for the MF-2000 Minifluorometer.

- (5) Check the calibration by testing the yellow vial.

Calibrations (in ppb) for Corn, Corn Meal, Corn/Soy Blend, Corn Germ Meal, Wheat, Sorghum, Soybeans, Flaking Corn Grits, Milled Rice, and Popcorn			
	<u>Series III</u>	<u>Series IV</u>	<u>MF-2000</u>
Red	150	140	*
Green	-3.0	-3.0	*
Yellow	75 \pm 5	70 \pm 5	66 - 74

Calibrations (in ppb) for Corn Gluten Meal and Corn Gluten Feed			
	<u>Series III</u>	<u>Series IV</u>	<u>MF-2000</u>
Red	110	100	*
Green	-3.0	-3.0	*
Yellow	55 \pm 5	50 \pm 5	66 - 74

*** Note: No values for the red and green calibrators.**

The MF-2000 does not give digital display values. Instead, a series of bar graph lights and the FGIS Aflatest overlay are used to read the yellow calibrator value. When the yellow vial is inserted, 10 bar graph lights should illuminate. This corresponds to a value between 66 - 74 ppb. Use the overlay to determine whether the value of the yellow vial is within FGIS specifications.

- (6) Record the result for the Yellow Vial.
- (7) If the value of the yellow calibration vial is not within FGIS specifications, repeat the calibration process (steps 2 through 4 listed above), then check the yellow vial again. If the reading for the Yellow Vial remains above or below FGIS specifications, contact the Mycotoxin Testing Group at TSD.
- (8) When the fluorometer is calibrated, place the standards back in the case and close tightly, and store away from any light source.
- (9) Check the calibration of the fluorometer at least once an hour or before analyzing any test samples if more than 1 hour time has elapsed since the last test using the Yellow Vial.

c. Calibration Standards.

(1) Maintenance.

The standard solutions in the three (3) standard vials (Red, Green, and Yellow) degrade slowly in the presence of light.

Since the plastic case containing the vials passes a small amount of light, it is recommended that both case and vials be stored in a cabinet or drawer away from all light except when calibrating or checking the calibration of the fluorometer.

Maintain two (2) sets of standards (two cases) at each location. Select and identify one set as the working standard, the other as the reference standard to be used to check the working standard every 14 days.

The degradation of the working set will occur gradually over a period of time, so anticipate expiration and requisition a replacement set in advance. (A sudden change in the reading of a vial indicates instrument instability, a cracked vial, or undue exposure of the vial to light.)

When one vial of a set expires, replace the entire set. About 2 months before the expected expiration of the working set, obtain a new set of standards from Vicam Co. When received, compare fluorometer readings of the new set with those of the existing reference set. If the difference between the two sets exceeds 3 ppb for any of the colors, notify TSD.

(2) Biweekly check of working standards.

Calibrate the fluorometer using the working set as described in "Calibration Procedures" (see section 8.3 b).

After calibrating the working set, remove the reference set from storage and test the 3 vials as described in section 8.4, b. The difference in readings of the two sets should not exceed the following limits:

<u>Red</u>	<u>Yellow</u>	<u>Green</u>
± 10 ppb	± 5 ppb	± 2 ppb

If the difference between the working and reference sets exceeds the tolerances, discard the working set. Begin using the old reference set as the working set, and use the new set as the reference set. Keep a permanent record of all calibration verification data.

8.4 SOLUTION TESTING

The distilled/deionized water, dilute developer solution, and HPLC grade methanol must be tested for background fluorescence before use. After calibrating the fluorometer perform the following:

a. Methanol.

Place 2.0 ml of HPLC grade methanol into a clean cuvette. Place the cuvette in the calibrated fluorometer. The displayed reading should be between -3.0 and +1.0. If the reading is positive and greater than 1.0, replace the methanol.

b. Water.

Dispense 2.0 ml of deionized/distilled water into a clean cuvette. Place the cuvette in the calibrated fluorometer. The digital display reading should be between -3.0 and +1.0. If the reading is positive and greater than 1.0, take action to assure a pure water supply.

c. Developer Solution.

Combine 1.0 ml of dilute developer solution and 1.0 ml of HPLC grade methanol in a clean cuvette. Place the cuvette in the calibrated fluorometer. The digital display reading should be between -3.0 and +1.0.

If the reading is positive and greater than 1.0, check each reagent separately to determine which reagent is causing the problem and replace it.

8.5 TEST PROCEDURES

a. Procedures for Testing Corn, Corn Meal, Corn/Soy Blend, Flaking Corn Grits, Milled Rice, Popcorn, Sorghum, and Soybeans.

Note: All aflatoxin tests for rice are performed on a milled rice basis. Consequently, rough rice or brown rice require milling before analysis. Mill rough rice or brown rice according to the procedures in the Rice Handbook.

(1) Extraction.

- (a) Place 50 g of ground sample into blender jar.
- (b) Add 5 grams of analytical, USP grade sodium chloride (NaCl) or food grade un-iodized salt.
- (c) Add 100 ml of the 80/20 methanol/water extraction solution.
- (d) Cover jar and blend at high speed for 1 minute.
- (e) Remove the cover and pour the extract into a filter paper (Whatman 2V folded or S&S 591 24 cm pleated or equivalent) supported in a clean funnel.
- (f) Collect the filtrate in a clean beaker labeled with the sample identification.
- (g) After collecting approximately 25 ml of extract, carefully dispose of the filter paper and its contents.
- (h) Pipette 5 ml of filtered extract into a clean beaker.

- (i) Add 10 ml of distilled/deionized water and mix thoroughly.
- (j) Filter the diluted extract through a glass microfibre filter (Vicam Cat. # 31955) supported by a small, clean funnel. Fold the glass microfibre filter gently without making a sharp crease to avoid breaking the glass microfibre filter.
- (k) Immediately proceed with the Aflatest Affinity Column procedure.

Note: If this diluted filtrate turns cloudy, refilter using a new glass microfibre filter before proceeding with the analysis.

(2) Affinity Column.

- (a) Prepare an Aflatest-P affinity column for use by removing both end caps and gently shaking the buffer solution from the top of the column.
- (b) Using an Eppendorf pipette, add 1.0 ml of the filtered dilute extract to the top of the Aflatest column.
- (c) Attach the column to the washing device (either a syringe barrel or an air pumping station) and pass the filtered extract through the column using a steady positive pressure. Maintain a flow rate of approximately 1 drop per second.

Note: Sample analysis using these procedures can be greatly simplified by the use of a small aquarium air pump to provide the needed air pressures for loading, filtering, and washing the various extracts.

- (d) After the extract has completely passed through the Aflatest column, add 1 ml of deionized or distilled water to the column and again apply a steady positive pressure to pass the wash water through the column. (If a syringe barrel rather than the pumping station is used, detach the column and pipette 1 ml of deionized or distilled water into the column headspace.) Reattach the column to the syringe barrel and apply pressure to pass the water through the column.

- (e) Repeat the water wash in step (d) above.
 - (f) After the second wash has passed through the column, place a clean cuvette under the outlet of the column. Only 12 x 75 mm borosilicate glass tubes should be used for cuvettes (Vicom Cat. # 34000 or equivalent). Use care when handling the cuvette to keep the optical surface clean and free of lint, fingerprints, etc.
 - (g) Dispense 1.0 ml of HPLC grade methanol into the column. If a syringe barrel rather than the pumping station is used, detach the column, pipette 1 ml of methanol directly into the column headspace, and replace the column.
 - (h) Apply a steady pressure to elute/pass the methanol through the column and collect all of the methanol eluate in the cuvette. Maintain pressure to collect the methanol at a rate of approximately 1 drop per second.
 - (i) Add 1.0 ml of dilute Aflatest Developer Solution directly to the sample eluate solution in the cuvette and mix well (about 5 seconds).
 - (j) **Immediately** place the cuvette in a calibrated fluorometer.
- (3) Reading, Recording, and Certifying Test Results.
- (a) Record the digital readout (Series III and IV) or corresponding bar graph value (MF-2000) as total ppb.
 - (b) Report all results on the pan ticket and the inspection log to the nearest whole ppb.
 - (c) Sample results over 300 ppb are reported as >300 ppb unless a supplemental analysis is performed.
 - (d) Refer to the Certification section of the handbook for more detailed certification procedures.

(4) Supplemental Analysis.

To determine and report an aflatoxin level higher than 300 ppb, the filtered test sample extract must be diluted so that a value between 5 ppb and 300 ppb is obtained. The final aflatoxin concentration is calculated by multiplying the results obtained with the diluted extract by the dilution factor.

- (a) Using an Eppendorf pipette, add 0.5 ml (instead of 1.0 ml) of the filtered diluted extract to the top of the Aflatest column headspace. (See section 8.5 a (2) (b).)
- (b) Analyze the filtered extract as a normal sample.
- (c) Multiply the analytical results obtained by 2 to obtain the actual aflatoxin concentration. For example, if 240 ppb was the sample value obtained using the diluted test sample procedure, the actual concentration in the original sample was 480 ppb.

Example:	Diluted test sample extract result	240 ppb
	Dilution factor	<u>x 2</u>
	Actual aflatoxin concentration	480 ppb

Note: Laboratories may dilute samples as a first step if levels typically observed exceed 300 ppb and the applicant requests certified results above the range of the test kit.

b. Procedures for Testing Corn Germ Meal and Wheat.

(1) Extraction.

- (a) Place 50 g of ground sample into blender jar.
- (b) Add 10 grams of analytical, USP grade sodium chloride (NaCl) or food grade un-iodized salt.
- (c) Add 200 ml of the 80/20 methanol/water extraction solution.
- (d) Cover jar and blend at high speed for 1 minute.

- (e) Remove the cover and pour the extract into a filter paper (Whatman 2V folded or S&S 591 24 cm pleated or equivalent) supported in a clean funnel.
- (f) Collect the filtrate in a clean beaker labeled with the sample identification.
- (g) After collecting approximately 25 ml of extract, carefully dispose of the filter paper and its contents.
- (h) Pipette 5 ml of filtered extract into a clean beaker.

Note: If the solution filtration is slow (i.e., more than two minutes are required to collect 5 ml of filtrate), withdraw 5.0 ml of the clearest liquid from the top of the material held in the funnel (see step (e) above) and transfer it to a clean container.

- (i) Add 10 ml of distilled/deionized water and mix thoroughly.
- (j) Filter the diluted extract through a glass microfibre filter (Vicom Cat. # 31955) supported by a small, clean funnel. Fold the glass microfibre filter gently without making a sharp crease to avoid breaking the glass microfibre filter.
- (k) Immediately proceed with the Aflatest Affinity Column procedure.

Note: If this diluted filtrate turns cloudy, refilter using a new glass microfibre filter before proceeding with the analysis.

(2) Affinity Column.

- (a) Prepare an Aflatest-P affinity column for use by removing both end caps and gently shaking the buffer solution from the top of the column.
- (b) Using an Eppendorf pipette, add 1.0 ml of the filtered dilute extract to the top of the Aflatest column.

- (c) Attach the column to the washing device (either a syringe barrel or an air pumping station) and pass the filtered extract through the column using a steady positive pressure. Maintain a flow rate of approximately 1 drop per second.

Note: Sample analysis using these procedures can be greatly simplified by the use of a small aquarium air pump to provide the needed air pressures for loading, filtering, and washing the various extracts.

- (d) Using an Eppendorf pipette, add 1.0 ml of the filtered dilute extract to the top of the Aflatest column.
- (e) Attach the column to the washing device (either a syringe barrel or an air pumping station) and pass the filtered extract through the column using a steady positive pressure. Maintain a flow rate of approximately 1 drop per second.
- (f) After the extract has completely passed through the Aflatest column, add 1 ml of deionized or distilled water to the column and again apply a steady positive pressure to pass the wash water through the column. (If a syringe barrel rather than the pumping station is used, detach the column and pipette 1 ml of deionized or distilled water into the column headspace.) Reattach the column to the syringe barrel and apply pressure to pass the water through the column.
- (g) Repeat the water wash in step (f) listed above.
- (h) After the second wash has passed through the column, place a clean cuvette under the outlet of the column. Only 12 x 75 mm borosilicate glass tubes should be used for cuvettes (Vicam Cat. # 34000 or equivalent). Use care when handling the cuvette to keep the optical surface clean and free of lint, fingerprints, etc.
- (i) Dispense 1.0 ml of HPLC grade methanol into the column. If a syringe barrel rather than the pumping station is used, detach the column, pipette 1 ml of methanol directly into the column headspace, and replace the column.

- (j) Apply a steady pressure to elute/pass the methanol through the column and collect all of the methanol eluate in the cuvette. Maintain pressure to collect the methanol at a rate of approximately 1 drop per second.
- (k) Add 1.0 ml of dilute Aflatest Developer Solution directly to the sample eluate solution in the cuvette and mix well (about 5 seconds).
- (l) **Immediately** place the cuvette in a calibrated fluorometer.

(3) Reading, Recording, and Certifying Test Results.

- (a) Record the digital readout (Series III and IV) or corresponding bar graph value (MF-2000) as total ppb.
- (b) Report all results on the pan ticket and the inspection log to the nearest whole ppb.
- (c) Sample results over 300 ppb are reported as >300 ppb unless a supplemental analysis is performed.
- (d) Refer to the Certification section of the handbook for more detailed certification procedures.

c. Procedures for Testing Corn Gluten Meal and Corn Gluten Feed.

(1) Extraction.

- (a) Place 50 g of ground sample into blender jar.
- (b) Add 5 grams of analytical, USP grade sodium chloride (NaCl) or food grade un-iodized salt.
- (c) Add 250 ml of 60 percent methanol/40 percent water extraction solution to blender jar. The 60/40 percent methanol/water solution is prepared by mixing 600 ml HPLC grade methanol with 400 ml distilled/deionized water.
- (d) Cover jar and blend at high speed for 1 minute.

- (e) Remove the cover and pour the extract into a filter paper (Whatman 2V folded or S&S 591 24 cm pleated or equivalent) supported in a clean funnel.
 - (f) Collect the filtrate in a clean beaker labeled with the sample identification.
 - (g) After collecting approximately 25 ml of extract, carefully dispose of the filter paper and its contents.
 - (h) Pipette 10 ml of filtered extract into a clean beaker.
 - (i) Add 20 ml of distilled/deionized water and mix thoroughly.
 - (j) Filter the diluted extract through a glass microfibre filter (Vicam Cat. # 31955) supported by a small, clean funnel. Fold the glass microfibre filter gently without making a sharp crease to avoid breaking the glass microfibre filter.
 - (k) Load 6-8 ml of the filtrate from step (j) above into a 10 ml plastic syringe barrel fitted with 0.22 micron nylon syringe disk filter (Fisher Scientific Corporation CAMEO II Cat. No. DDN 02T2550, Gelman Cat. No. 09-730-191, or Corning Cat. No. 09-754-22).
 - (l) Apply enough air pressure to syringe barrel to produce a flow of approximately 1 drop per second through disk filter and collect a minimum of 5 ml of filtrate in a clean test tube. Discard filter disk.
- (2) Affinity Column.
- (a) Prepare an Aflatest-P affinity column for use by removing both end caps and gently shaking the buffer solution from the top of the column.
 - (b) Using a 1.0 ml Eppendorf pipette, load 4.0 ml of refiltered extract from step (l) above into the barrel of a 10 ml glass syringe to which an Aflatest-P column is attached.

- (c) Apply pressure so that the extract passes through the column at 1 to 2 drops per second. Remove syringe barrel from column. Fill column with distilled water. Reattach syringe barrel to column.
 - (d) Fill syringe barrel with 10 ml of distilled/deionized water and pass through column at a flow rate of approximately 2 drops per second. Allow all of wash water to pass through column.
 - (e) Repeat column wash with another 10 ml of deionized/distilled water.
 - (f) Elute aflatoxin from Aflatest-P column with 1 ml HPLC grade methanol and collect sample eluate solution in glass cuvette.
 - (g) Add 1 ml of fresh, dilute Aflatest developer solution directly to the eluate in cuvette and mix well.
 - (h) **Immediately** place the cuvette in a calibrated fluorometer.
- (3) Reading, Recording and Certifying Test Results.
- (a) Record the digital readout (Series III and IV) as total ppb. **To determine the aflatoxin concentration using the MF-2000 fluorometer, read the corresponding bar graph value and multiply by 0.73 for actual ppb.**
 - (b) Report all results on the pan ticket and the inspection log to the nearest whole ppb.
 - (c) Sample results over 300 ppb are reported as >300 ppb.
 - (d) Refer to the Certification section of the handbook for more detailed certification procedures.

NOTE: Rinse both glass and plastic syringe barrels with approximately 10 ml of distilled/deionized water each before analyzing next sample.

8.6 CLEANING LABWARE

a. Negative Tests (# 20 ppb).

(1) Labware.

Prepare a solution consisting of dishwashing liquid and water. Completely submerge the used glassware, funnels, beakers, etc., wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Place materials in a garbage bag for routine trash disposal.

b. Positive Tests (> 20 ppb).

(1) Labware.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water (e.g., 100 ml bleach to 1,000 ml water). Completely submerge the used glassware, funnels, beakers, etc., and soak for at least 5 minutes. Remove items from the bleach/water solution, submerge in a dishwashing liquid/water solution, wash thoroughly, then rinse with clean water before reusing.

(2) Disposable Materials.

Prepare a bleach solution consisting of 1 part bleach to 10 parts water in a plastic pail labeled "bleach solution." Soak disposable materials, such as used columns, cuvettes, vials, test kit components, etc., for at least 5 minutes. Pour off the liquid down the drain and place the materials in a garbage bag and discard.

8.7 WASTE DISPOSAL

a. Negative Results (# 20 ppb).

If the test result is negative (equal to or less than 20 ppb), discard the filter paper and its contents (ground material) into a plastic garbage bag for disposal. Dispose of any remaining liquid filtrate in the chemical waste container.

b. Positive Results (> 20 ppb).

If the result is positive (more than 20 ppb), the ground portion remaining in the filter paper must be decontaminated prior to disposal. After disposing of the remaining filtered extract in the chemical waste container, filter approximately 50 ml of bleach through the filter containing the ground portion and allow to drain. Discard the filter paper and its contents (ground portion) into a plastic garbage bag for disposal. The bleach rinse filtrate collected may be treated as a non-hazardous solution and disposed of by pouring down the drain.

8.8 EQUIPMENT and SUPPLIES

- a. Fluorometer - Romer model RL-100, Vicam Series III and IV, or Vicam model MF-2000.
- b. Fluorometer calibration standards. (Vicam # 33030)
- c. Cuvette Rack. (Vicam # 21010)
- d. Pump assembly stand, double. (Vicam # 21030)
- e. Syringe, glass 10 ml. (Vicam # 34010)
- f. Syringe hand pump with coupling. (Vicam #36030)
- g. Automatic pipettor (1 ml capacity for methanol). (Vicam #20501)
- h. Automatic pipettor (1 ml capacity for developer). (Vicam #20600)
- i. Graduated cylinders - 25 ml, 100 ml, and 250 ml capacity.
- j. Aflatest-P columns. (Vicam # 12022)
- k. Cuvettes, disposable 12 x 75 mm borosilicate glass tube. (Vicam # 34000)
- l. Disposable beakers. (Vicam # 36010)
- m. Glass microfibre filter paper -Whatman 934-AH. (Vicam # 31955)
- n. Small plastic funnels.

- o. Wash bottles or spray bottles.
- p. Box of Kim Wipes (small size sheets).
- q. HPLC grade methanol.
- r. Aflatest developer solution. (Vicam # 32010)
- s. Balance.
- t. Sample Grinder.
- u. Distilled/deionized water.
- v. Aflatest developer solution. (Vicam #32010)
- w. USP grade sodium chloride (NaCl) or food grade un-iodized salt.

8.9 STORAGE CONDITIONS

- a. Affinity Columns - Store at room temperature (64° to 86° F).
- b. Calibration Vials - Store in a cabinet or drawer away from all light, except when in use.
- c. Developer Concentrate - Store in a tightly closed bottle in a cool, dry, well ventilated area and away from sunlight, combustible materials, and incompatible materials.