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1. Purpose

The mycotoxin analysis training program provides background and training in the analysis of mycotoxins in foods and feeds. The Office of Training, Education and Development (OTED) provides the New Hire Laboratory Analyst Training curriculum (Bingo card) which specifies sections of the ORA Lab Manual as part of the New Hire Curriculum. This section describes

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mycotoxin analyses for new hire training and provides suggested on-the-job training of selected techniques.

The Center for Food Safety and Applied Nutrition (CFSAN) and the Center for Veterinary Medicine (CVM) are responsible for establishing the inspectional and analytical guidelines associated with the FDA mycotoxin programs. The two mycotoxin Compliance Programs, 7307.001 Mycotoxins in Domestic and Imported Foods and 7371.003 Feed Contaminants Program, are found in the Compliance Program Guidance Manual (CPGM).

This training chapter introduces the trainee to regulatory analyses of mycotoxins in the FDA, safety precautions, sample preparation, analytical separation techniques and instrumentation. The trainee should discuss each exercise in advance with the trainer. If a laboratory does not perform analyses in that area, that portion of the training can be abbreviated or eliminated.

When the trainee has completed each exercise, the analysis, worksheet preparation, and answers to the questions should be discussed thoroughly. This provides the analyst the opportunity to demonstrate that they are proficient in the technique and instrumentation.

2. Scope

The incumbent is a regulatory scientist in an FDA/ORA field laboratory and is expected to bring full professional competence in their discipline to bear in carrying out analyses and interpreting the significance of test results. The incumbent is assigned scientific analyses on a wide range of samples including those that are difficult, complex, or unusual.

These responsibilities support the FDA and its mission to support public health through the regulation of mycotoxins in foods and feeds under the jurisdiction of the Federal Food, Drug, and Cosmetic Act (FD&C Act) and related Acts.

3. Responsibility

A. Trainer

1. Coordinates training with Supervisor(s) and Trainee.
2. Works with trainees to ensure completion of all training needed to meet their regulatory responsibilities.

B. Trainee

1. Completes required training within specified timeframes.

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2. Reports training received and submits documentation for training to supervisor.
- C. Supervisor
1. Implements and reviews training records.
- D. Quality Management or designee
1. Maintains employee competency records for staff.

4. Background

Mycotoxins are natural poisons produced by fungi as secondary metabolites. Foods and animal feeds may become contaminated with mycotoxins as a result of mold growth during harvest or storage. Three genera are responsible for the majority of the mycotoxins with which FDA is concerned: *Aspergillus*, *Penicillium*, and *Fusarium*. Of the numerous mycotoxins elaborated by these fungi, FDA is actively concerned with aflatoxins, fumonisins, trichothecenes (i.e. DON, HT-2, T-2), ochratoxin A, patulin, and zearalenone. The potential for a product to contain a naturally incurred mycotoxin depends on whether the product contains and supports the growth of a mycotoxin-producing mold species, and whether the optimum temperature and humidity are present.

In addition to giving instructions to the FDA field (the laboratory, investigation, and compliance branches) on how to accomplish their mycotoxin assignments, the FDA mycotoxin compliance programs give introductory information about these mycotoxins and the products which are susceptible to contamination by them (see Section 5 References A-B). The compliance programs also list the analytical methods for each mycotoxin. For more information on the occurrence, health impacts and sources of mycotoxins, see Section 5 References C-K.

An extensive amount of information and guidance about mycotoxins is available on the internet. One example, CFSAN's Bad Bug Book, can be found in Section 5 Reference L and provides many details about aflatoxins. There is a corresponding FDA Compliance Policy Guide (CPG) for each of the mycotoxins that have an established action level. For example, CPG Sec. 510.150 Apple Juice, Apple Juice Concentrates, and Apple Juice Products – Adulteration with Patulin (Section 5 Reference M) establishes the action level for patulin in apple juice, apple juice concentrate, and apple juice products. CPGs give guidance for recommending legal action, such as detention of imports. They specify criteria for recommending action, the wording of the legal charge, and which analyses are needed. In addition to regulatory action levels, the FDA has set guidance and advisory levels for other mycotoxins in order to

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protect food and feed safety and to guide food and feed industries. These additional regulatory limits can be found in the mycotoxin compliance programs or in FDA guidance for industry documents (Section 5 References N-O).

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6. Procedure

6.1. Safety Precautions

General laboratory safety precautions concerning toxic substances addressed in the ORA Laboratory Manual (Volume III, Section 2, Environmental Health and Safety) and safety considerations regarding mycotoxins should be understood before any laboratory work is conducted. Aflatoxins are carcinogenic to humans and are highly toxic, especially aflatoxin B1. Trichothecenes are potent dermal irritants and orally toxic. Never dispose of these or any other mycotoxins by pouring their aqueous solutions down the drain or their organic solutions into a waste container. Chemical inactivation of

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all mycotoxins should be performed using a sodium hypochlorite solution of the appropriate strength and volume in accordance with the laboratory's chemical hygiene plan for disposal and treatment of spills.

Weighing and transferring mycotoxins in dry form should be avoided. The electrostatic nature of a number of the mycotoxins in dry form results in a tendency for them to be easily dispersed in the working area and to be attracted to exposed skin and clothes. If mycotoxin standards are received in a dry form, they should be dissolved in a solvent while in the original container using the label statement of weight as a guide, and the concentrations should be determined spectrophotometrically. Containers of mycotoxin standard solutions should be tightly capped, and their weights should be recorded for future reference before wrapping them in foil and storing them in a freezer (see Section 5 Reference P, Chapter 49, 971.22E). Keeping records of the weights of stored solutions will provide a means of determining if the solutions have become concentrated during storage. To store the material as a film, the solvent should be removed at a moderately elevated temperature under a stream of nitrogen, or by rotary evaporation before freezer storage.

6.2. Sample Preparation

The objective of sample preparation in trace analysis is to obtain a sample of convenient size (usually 1-100 g) whose composition accurately reflects the composition of the bulk sample from which it was taken (usually 2-25 kg). That sample composite needs to be representative of the entire sample collected for analysis. The nature of mycotoxin contamination makes this objective more difficult to attain than for most other types of trace analyses. Because the molds that produce mycotoxins do not grow uniformly on the substrate, contamination of the commodity is usually far from uniform (See Section 5 Reference P, Chapter 49, Method 977.16). However, when food is prepared from that commodity, the mycotoxin present in perhaps only a small portion of the commodity may be spread throughout the entire lot of food.

Sample preparation can be divided into three steps: sample size reduction, particle size reduction, and mixing. Sample size reduction is dividing or splitting the sample while maintaining a random distribution between the split portions in order to ensure these portions remain representative of the entire sample. One of the acceptable methods for sample size reduction is using a sample splitter such as a Jones riffle divider/splitter. Particle size reduction can be accomplished using a vertical cutter/mixer (VCM), cutting mill, rotor mill, meat grinder, or other equivalent equipment. Mixing can be accomplished using a VCM, polytron homogenizer, laboratory blender, or other equivalent equipment. All three steps are not carried out for all samples and if so, not necessarily in the order given. In some cases, the grinding equipment

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combines the particle size reduction with the mixing step. In other cases, the steps must be performed separately because the grinding operation does not thoroughly mix the sample composite. Choose the proper equipment for each sample in order to obtain a properly ground and mixed sample, called a “composite”. Following compositing of regulatory samples, two equally representative portions of the composite should be placed in two containers – one for an “Original” analysis, and one for a “Check” analysis. An additional representative portion of composite may also be saved as a “Reserve” portion. The remainder of the sample composite is disposed. In the case of a violative sample, the remaining original and check portions, as well as any 702(b) portion, should be sealed and returned to the Sample Custodian as the reserve sample.

The samples themselves may be grouped in two categories: those that are moist or relatively high in oil content and yield a paste when ground (e.g. peanuts, tree nuts, dates), and those that are dry and yield a powder or dry particles when ground (e.g. corn, small grains). For contextual guidance on the preparation of nut samples, see Section 5 References Q-S. Dry samples present a different set of problems that come from two sources. The kernel or grain of a dry commodity is often not the same hardness and density throughout, and the mold may grow or produce mycotoxin preferentially on one part of the kernel or grain. When the sample is ground, the softer portion of the kernel or grain is often ground to a smaller particle size than the harder portion. These smaller particles migrate to the bottom of the sample container when the sample is handled. Consequently, the sample withdrawn for analysis may no longer be representative, and the analytical result may be inaccurate. To minimize these problems, one needs to mix the sample thoroughly and withdraw the portion for analytical determination as soon as practical after mixing. The trainer will further discuss the selection and proper use of the sample preparation equipment in the laboratory.

Proper storage of sample composites is critical for mycotoxins analysis in order to ensure sample integrity. Mycotoxin sample composites should not be stored in conditions of high temperature or humidity and should be frozen for extended storage to prevent mold growth or any additional spoilage. *Aspergillus* species will not grow under refrigeration or freezing, however some *Penicillium* and *Fusarium* species can grow under refrigeration and just below freezing. Sample composites for patulin analysis should always be stored frozen to prevent any possible fermentation caused by spoilage as fermentation can destroy patulin in juices (see Section 5 Reference T).

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6.3. Exercises

The purpose of these exercises is to familiarize the analyst with the equipment and procedures used to obtain a representative composite and the methodology used for the isolation, determination, and confirmation of the most commonly encountered mycotoxins.

The trainer will instruct each trainee in the proper sample preparation procedure for each commodity. The trainer should obtain bulk samples of 2-25 kg each of whole-kernel corn, shelled peanuts, and wheat or barley. Sufficient amounts of each prepared commodity should be given to each trainee for use in the following exercises.

Note: Training on all the various mycotoxin methods is not required if the laboratory does not perform those analyses. Even though the official method for confirmation of identity of aflatoxin B₁ is by chemical derivative, confirmation of identity for all mycotoxins is performed by Liquid Chromatography-Mass Spectrometry (LC-MS) for violative samples when MS instrumentation is available.

A. Questions

1. What is the difference between mycotoxin compliance and surveillance samples?
2. Using the IOM Sample Schedule, Chart 6, Mycotoxin Sample Sizes (see Section 5 Reference U), determine whether the following samples are surveillance or compliance samples:
 - a. 12 subs x 1 lb. sample of chunky peanut butter (=12 lb. sample)
 - b. 12 subs x 1 lb. sample of creamy peanut butter (=12 lb. sample)
 - c. 10 subs x 1 lb. sample of shelled almonds (=10 lb. sample)
 - d. 50 subs x 1 lb. sample of shelled almonds (=50 lb. sample)
 - e. 10 subs x 1.5 lb. sample of almond paste (=15 lb. sample)
 - f. 10 subs x 1 lb. sample of corn (=10 lb. sample).

6.3.1. Aflatoxins B₁, B₂, G₁, and G₂

Aflatoxins are metabolic products of the molds *Aspergillus flavus* and *Aspergillus parasiticus* and may occur in food as a result of mold growth in a number of susceptible commodities, including peanuts, corn, Brazil nuts, pistachio nuts, pumpkin seeds, cotton seeds, and watermelon seeds. Other nuts, grains, and seeds are susceptible but less prone to contamination with aflatoxins. Because aflatoxins are known carcinogens to humans and animals,

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the presence of aflatoxins in foods should be restricted to the minimum levels practically attainable using processing techniques.

Results for aflatoxins found in in-shell nuts and seeds are calculated and reported on an edible basis, assuming that all of the aflatoxin is in the edible portion of the product and none is in the shell. For nuts, this is called a nutmeat basis. The units are µg/kg or ppb.

Liquid Chromatography (LC-Fluorescence), Immunoaffinity Method:

A. Assignments

1. Prepare standard solutions of aflatoxins B₁, B₂, G₁, and G₂ as described in AOAC 991.31D (see Section 5 Reference P).
2. Weigh four 25 g portions of an assigned sample composite into blender cups with covers. Two of these samples are to be spiked at the same level with aflatoxins by the trainer. Analyze all four samples as described in AOAC 991.31 (see Section 5 Reference P). Identify and calculate the quantities of the aflatoxins found. Be prepared to discuss the results and any problems encountered.

Note: Trainer may use other accepted methods for aflatoxins B₁, B₂, G₁, and G₂ analysis.

B. Questions

1. What are aflatoxins? How do they affect humans and animals?
2. Why is the preparation of a representative or homogeneous sample so critical in this determination?
3. How do the names of these four aflatoxins (B₁, B₂, G₁, and G₂) correlate with their appearance and chromatographic pattern if they are displayed on a TLC plate?
4. In what order do these four aflatoxins (B₁, B₂, G₁, and G₂) show up on the chromatogram using this method (reversed-phase chromatography)?
5. Why is the dilution of the sample critical before placing the sample solution on the immunoaffinity column?
6. Explain the principle of reversed-phase HPLC.
7. Describe another way to confirm aflatoxin B₁ or G₁ found in a sample other than by TFA derivative formation.
8. How many grams of ground in-shell pistachios should be weighed to obtain 25 g of nutmeat for analysis?

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9. What references would be consulted first for information on new or improved techniques for the determination of aflatoxins or other mycotoxins?

6.3.2. Aflatoxin M₁

Aflatoxin M₁ is produced by lactating animals consuming aflatoxin contaminated feed. Aflatoxin B₁ is metabolized into aflatoxin M₁. Monitoring aflatoxin M₁ is an important part of the food safety program because milk is an important basic food, particularly for infants and children.

Liquid Chromatography (LC-Fluorescence), Immunoaffinity Method:

A. Assignments

1. Prepare standard solutions of aflatoxin M₁ as described in *J. AOAC Int.* 99, 2016, 174-179 (see Section 5 Reference V).
2. Weigh four 10 g portions of an assigned milk sample composite into an appropriate container. Two of these samples are to be spiked at the same level by the trainer. Analyze all four samples as described in *J. AOAC Int.* 99, 2016, 174-179 (see Section 5 Reference V).

Note: Trainer may use other accepted methods for the analysis of aflatoxin M₁.

B. Questions

1. What is the acceptable level of aflatoxin M₁ in milk?
2. What is the purpose of centrifugation at low temperatures?
3. Why are sodium chloride (NaCl) and magnesium sulfate (MgSO₄) used in this method?
4. In using the immunoaffinity method are antibodies on the immunoaffinity column (IAC) monoclonal or polyclonal?

6.3.3. Ochratoxin A

Ochratoxin A is a naturally occurring nephrotoxic fungal metabolite produced by certain species of the genera *Aspergillus* and *Penicillium*. It is mainly a contaminant of cereals (corn, barley, wheat, and oats), and has been found in edible animal tissues as well as in human sera and milk. It has also been found in raisins, currants, and green coffee. Studies indicate that this toxin is carcinogenic in mice and rats. It can be destroyed during the processing and cooking of food, therefore the implication of health risk to human health and safety is considered less than that of aflatoxins.

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Liquid Chromatography (LC-Fluorescence), Immunoaffinity Method:

A. Assignments

1. Prepare standard solutions of ochratoxin A as described in AOAC 2000.03 (see Section 5 Reference P) or *J. AOAC Int.* 82, 1999, 85-89 (see Section 5 Reference W).
2. Weigh four 25 g portions of an assigned sample composite into blender cups with covers. Two of the samples are to be spiked at the same level by the trainer. Analyze all four samples by AOAC 2000.03 or *J. AOAC Int.* 82, 1999, 85-89 (see Section 5 References P or W).
3. Confirm the identity of the ochratoxin A found in one of the spiked samples by using LC-MS.

Note: Trainer may use other accepted methods for ochratoxin A analysis.

B. Questions

1. What is an advantage of using an immunoaffinity column method?
2. Why is a buffer solution of pH 7.4 needed for this method?

6.3.4. Zearalenone

Zearalenone is an estrogenic mycotoxin produced by fungus *Fusarium graminearum*. Zearalenone can interfere with conception, ovulation, implantation, fetal development, and the viability of newborn animals. This toxin can occur in corn and corn-based feeds. It is also found in other important crops such as wheat, barley, sorghum, and rye.

Liquid Chromatography (LC-Fluorescence), Immunoaffinity Method:

A. Assignments

1. Prepare standards of zearalenone as described in LIB 4488 (see Section 5 Reference X).
2. Weigh four 25 g portions of an assigned sample composite into blender cups with covers. Two of these samples are to be spiked at the same level by the trainer. Analyze all four samples as described in LIB 4488 (see Section 5 Reference X).
3. Confirm the identity of zearalenone found in both spiked samples as described in LIB 4488 (see Section 5 Reference X).

Note: Trainer may use other accepted methods for zearalenone analysis.

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B. Questions

1. What is the method detection level (MDL) and limit of quantitation (LOQ) for this method?
2. What other mycotoxins can be separated using this method and how?

6.3.5. Trichothecenes

The trichothecene mycotoxins (i.e. DON, HT-2, T-2) are a group of closely related, secondary metabolites produced by various strains of *Fusarium*, *Trichoderma*, *Myrothecium*, and some other fungi. Some of the most common toxicological effects caused by these toxins are necrosis, diarrhea, and vomiting. These toxins occur in corn, wheat, barley, oats, rice, rye, and other crops.

Deoxynivalenol (DON/Vomitoxin), LC-UV (SPE or Immunoaffinity Method):

A. Assignments

1. Prepare standards of deoxynivalenol (DON) as described in *J. AOAC Int.* 79, 1996, 883-888 or *J. AOAC Int.* 98, 2015, 806-809 (see Section 5 References Y-Z).
2. Weigh four 25 g portions of an assigned sample composite into blender cups with covers or other appropriate containers. Two of these samples are to be spiked at the same level by the trainer. Analyze all four samples for DON as described in *J. AOAC Int.* 79, 1996, 883-888 or *J. AOAC Int.* 98, 2015, 806-809 (see Section 5 References Y-Z).

Note: Trainer may use other accepted methods for DON analysis.

B. Questions

1. Can conclusive identification be achieved by equivalent HPLC retention times (RT) between a sample peak and the DON standard peak alone? What other method or instrument could be used for confirmation of identity of the compound found in the sample extract?
2. Does DON have affinity to the SPE cleanup column packing used in *J. AOAC Int.* 79, 1996, 883-888 (see Section 5 References Y)?
3. Why is a combination of isocratic and step gradient conditions used in *J. AOAC Int.* 79, 1996, 883-888 (see Section 5 References Y)?
4. Is DON stable during most processing procedures including baking?

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6.3.6. Patulin

Patulin is a mycotoxin that is produced by certain species of *Penicillium*, *Aspergillus*, and *Byssosclamyces* molds that may grow on variety of foods including fruit, grains, and cheese. Patulin has been found to occur in a number of foods including apple juice, apples, and pears. Patulin contamination is primarily associated with damaged and rotting fruits and fruit juices made from poor quality fruits.

Samples of frozen concentrate or bulk concentrate should be diluted either as per the labeling instructions for dilution or to a Brix value of 11.5° (single strength) before analysis. See Section 5 Reference P, Chapter 44, AOAC 932.14C, Solids in Syrup.

Liquid Chromatography (LC-UV):

A. Assignments

1. Prepare standard solutions of patulin as described in AOAC 995.10C (see Section 5 Reference P) at the time of analysis.
2. Measure 5 mL of an assigned apple juice sample composite into four 20 x 150 mm glass culture tubes or other appropriate containers. Two of these samples are to be spiked at the same level by the trainer. Analyze all four samples as described in AOAC 995.10 (see Section 5 Reference P).

Note: Trainer may use other accepted methods for patulin analysis.

B. Questions

1. Is there another way that the extraction could have been carried out? Discuss the advantages of the proposed way and the official method.
2. What instruments can be used for determining the Brix value of a concentrate of apple juice?
3. Why is the combined ethyl acetate sample extract washed with 1.5% Na₂CO₃?
4. What factors affect the stability of patulin standards and patulin sample extracts?
5. What is today's regulatory guidance for recommending legal actions against products collected for patulin analysis?
6. If the HPLC hydroxymethylfurfural (HMF) peak is close to the patulin peak, how may the separation of the two peaks be increased to obtain better resolution? Which kinds of products are

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more likely to have a significant HMF peak present in the chromatogram?

7. Why is it important to use anhydrous sodium sulfate to dry the ethyl acetate extract?

6.3.7. Fumonisin

Fumonisin are natural toxins produced by *Fusarium moniliforme*, and other *Fusarium* species; these molds are common natural contaminants of corn. Fumonisin have been linked to fatalities in horses and swine. Recent studies have demonstrated the presence of fumonisin in human foods, including corn meal and breakfast cereals. More than ten types of fumonisin have been isolated and characterized. Of these, fumonisin B₁ (FB₁), fumonisin B₂ (FB₂) and fumonisin B₃ (FB₃) are the major fumonisin of concern produced in nature.

Liquid Chromatography (LC-Fluorescence):

A. Assignments

1. Prepare standard solutions of fumonisin B₁, B₂, and B₃ as described in AOAC 995.15 (see Section 5 Reference P).
2. Weigh four 25 g portions of an assigned sample composite into blender cups with covers or other appropriate containers. Two of these samples are to be spiked at the same level by the trainer. Analyze all four samples as described in AOAC 995.15 (see Section 5 Reference P).

Note: Trainer may use other accepted methods for the analysis of fumonisin B₁, B₂, and B₃.

B. Questions

1. Why does the OPA reaction not work with fumonisin A₁ and A₂?
2. Why is there no screening procedure for fumonisin A₁ and A₂?
3. What condition occurs in horses upon ingesting high levels of fumonisin contaminated feed?
4. What are the recommended maximum levels of fumonisin in human foods and in animal feeds established by FDA?

6.3.8. Multi-Mycotoxins Method

The multi-mycotoxins method uses stable isotope dilution assay (SIDA) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) for the simultaneous determination of aflatoxins B₁, B₂, G₁, and G₂; deoxynivalenol;

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fumonisin B₁, B₂, and B₃; ochratoxin A; HT-2 toxin; T-2 toxin; and zearalenone in foods (see Section 5 Reference AA).

A. Assignments

1. Prepare standard solutions containing aflatoxins B₁, B₂, G₁, G₂; deoxynivalenol, fumonisins B₁, B₂, and B₃, ochratoxin A, T-2, HT-2, zearalenone, and their corresponding isotopically labeled internal standards as described in the LC-MS/MS multi-mycotoxins method (see Section 5 Reference AA).
2. Weigh four 2.00 ± 0.05 g portions of an assigned product that has been pre-processed as described in Section 5 Reference AA into 15 mL polypropylene centrifuge tubes or other appropriate containers. Two of these samples are to be spiked at the same level with mycotoxins by the trainer. Analyze all four samples as described in the LC-MS/MS multi-mycotoxins method. Identify and calculate the quantities of the compounds found. Be prepared to discuss the results and any problems encountered.

B. Questions

1. What is the molecular weight of each native mycotoxin analyzed for in this method according to Section 5 Reference AA?
2. Explain how LC-MS/MS definitively confirms the identity of a substance and why, even though liquid chromatography on its own is non-specific, it becomes specific when paired with MS/MS.
3. Describe how you would analyze a sample with a high level of one mycotoxin.

7. Glossary/Definitions

None

8. Records

None

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9. Supporting Documents

- A. Chapter IV of the FD&C Act. Retrieve from:
<https://www.fda.gov/regulatoryinformation/lawsenforcedbyfda/federalfooddrugandcosmeticactfdcact/default.htm>
- B. U.S. Food & Drug Administration, Center for Food Safety and Applied Nutrition. Compliance Program Guidance Manual. Compliance Program 7307.001 Mycotoxins in Domestic and Import Foods. Retrieve from:
<http://inside.fda.gov:9003/downloads/ProgramsInitiatives/Food/FieldPrograms/ucm019307.pdf>
- C. U.S. Food & Drug Administration, Center for Veterinary Medicine. Compliance Program Guidance Manual. Compliance Program 7371.003 Feed Contaminants Program. Retrieve from:
<https://www.fda.gov/media/74766/download>
- D. U.S. Food & Drug Administration, Office of Compliance. (2001, October 22). Compliance Policy Guide. CPG Sec. 510.150 Apple Juice, Apple Juice Concentrates, and Apple Juice Products - Adulteration with Patulin. Retrieve from: <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cpg-sec-510150-apple-juice-apple-juice-concentrates-and-apple-juice-products-adulteration-patulin>
- E. U.S. Food & Drug Administration, Office of Compliance. (1980, October 01). Compliance Policy Guide. CPG Sec. 527.400 Whole Milk, Lowfat Milk, Skim Milk – Aflatoxin M1. Retrieve from:
<https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cpg-sec-527400-whole-milk-lowfat-milk-skim-milk-aflatoxin-m1>
- F. U.S. Food & Drug Administration, Office of Compliance. (1980, October 01). Compliance Policy Guide. CPG Sec. 555.400 Foods - Adulteration with Aflatoxin. Retrieve from: <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cpg-sec-555400-foods-adulteration-aflatoxin>
- G. U.S. Food & Drug Administration, Office of Compliance. (1983, August 01). Compliance Policy Guide. CPG Sec. 570.200 Brazil Nuts - Adulteration with Aflatoxin. Retrieve from:
<https://www.fda.gov/media/72053/download>
- H. U.S. Food & Drug Administration, Office of Compliance. (1980, October 01). Compliance Policy Guide. CPG Sec. 570.375 Aflatoxin in Peanuts and Peanut Products. Retrieve from:
<https://www.fda.gov/media/72073/download>

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- I. U.S. Food & Drug Administration, Office of Compliance. (1980, October 01). Compliance Policy Guide. CPG Sec. 570.500 Pistachio Nuts – Aflatoxin Adulteration. Retrieve from:
<https://www.fda.gov/media/72084/download>
- J. U.S. Food & Drug Administration, Office of Regulatory Affairs, (current edition). Investigations Operations Manual (Chap. 4, Chart 6, Mycotoxin Sample Sizes). Retrieve from:
<https://www.fda.gov/media/75243/download>

10. Document History

Revision #	Status* (D, I, R)	Date	Author Name and Title	Approving Official Name and Title
1.3	R	09/01/2005	LMEB	LMEB
1.4	R	02/02/2010	LMEB	LMEB
1.5	R	02/06/2012	LMEB	LMEB
1.6	R	02/13/2014	LMEB	LMEB
02	R	05/27/2020	LMEB	LMEB

* - D: Draft, I: Initial, R: Revision

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11. Change History

Revision #	Change
1.3	Table of Contents – 7.6 title change; 7.7 added. Section 7.2 revised first sentence; 7.4.3 revised last sentence
1.4	NOTE: - revised last sentence 7.4.2 Aflatoxin M ₁ II. A. - revised 7.4.2 Aflatoxin M ₁ II. C. – added 4. 7.4.4 A. – added NOTE: 7.4.5 A. – changed Reference 35 to 27; added NOTE: 7.4.7 B. – deleted 1. 7.5 – updated web links 7.6 7.4.2 Aflatoxin M ₁ – added 4. 7.6 7.4.7 – deleted 1. Footer – updated web link
1.5	7.5 – 1. Compliance Program reference 7307.001 title updated; 3. removed compliance Program 7307.002 7.6 – 7.4.1 6. question and answer revised
1.6	Header – Division of Field Science to Office of Regulatory Science 7.5 15. – removed Division name
02	Revised into new template: Created new sections (1-3 & 9), changed formatting and revised contents throughout. Changes made to all sections, including extensive updating of methods, removal of outdated methods and updating of references. Added section 6.3.8 Multi-Mycotoxins Method.

12. Attachments

List of Attachments

Attachment A - Answer Key20

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Attachment A - Answer Key

6.3 Exercises

1. **What is the difference between mycotoxin compliance and surveillance samples?** Compliance samples are of sufficient size to be representative of the lot. The IOM Chart 6 allows smaller surveillance samples to be collected for screening. When any level of aflatoxin is found in a surveillance sample, a larger compliance size sample should be collected and analyzed. Regulatory action may be taken on compliance sample results, not surveillance size samples.
2. **Using the IOM Sample Schedule, Chart 6, Mycotoxin Sample Sizes (See Section 5 Reference U), determine whether the following samples are surveillance or compliance samples:**
 - a. **12 subs x 1 lb. sample of chunky peanut butter (=12 lb. sample)**
Surveillance sample
 - b. **12 subs x 1 lb. sample of creamy peanut butter (=12 lb. sample)**
Compliance sample
 - c. **10 subs x 1 lb. sample of shelled almonds (=10 lb. sample)**
Surveillance sample
 - d. **50 subs x 1 lb. sample of shelled almonds (=50 lb. sample)**
Compliance sample
 - e. **10 subs x 1.5 lb. sample of almond paste (=15 lb. sample)**
Compliance sample
 - f. **10 subs x 1 lb. sample of corn (=10 lb. sample)**
Compliance sample

6.3.1. Aflatoxins B₁, B₂, G₁, and G₂

1. **What are aflatoxins? How do they affect humans and animals?** Aflatoxins are a group of toxins produced by the molds, *Aspergillus flavus* and *Aspergillus parasiticus* as metabolic products. The toxins consist primarily of aflatoxin B₁, B₂, G₁, and G₂. M₁ is produced from B₁ in cow's milk. In humans and animals, aflatoxins cause liver cancer, suppress the immune system, are mutagenic, and decrease the production of milk and eggs in animals. Aflatoxin B₁ is the most potent carcinogen.
2. **Why is the preparation of a representative or homogeneous sample so critical in this determination?** Both sampling and laboratory preparation of a representative/homogeneous sample composite are important to obtain a

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laboratory result that reflects the level of toxin present in the lot of product. Since aflatoxins are produced by mold growth on the product, the contamination is not evenly distributed but occurs in pockets of high contamination. Sample preparation results in a finely ground, well mixed sample composite.

3. **How do the names of these four aflatoxins (B₁, B₂, G₁, and G₂) correlate with their appearance and chromatographic pattern if they are displayed on a TLC plate?** The names of those aflatoxins directly correlate to the order in which they would appear on a TLC plate and their color of fluorescence under UV-light. When displayed on a TLC plate, B₁ is the first blue spot, B₂ is the second blue spot, G₁ is the first green spot, and G₂ is the second green spot.
4. **In what order do these four aflatoxins (B₁, B₂, G₁, and G₂) show up on the chromatogram using this method (reverse phase chromatography)?** The four aflatoxins appear in the following order: G₂, G₁, B₂, and B₁.
5. **Why is the dilution of the sample critical before placing the sample solution on the immunoaffinity column?** To reduce the methanol concentration from the 70% of the extraction solvent to about 23%. Methanol is finally used as an elution solvent to remove the aflatoxin from the antibodies in the column. The immunoaffinity for aflatoxin is low in high concentrations of methanol.
6. **Explain the principle of reversed-phase HPLC.** Liquid chromatography is based on using the competing affinities of compounds between a stationary phase and a mobile phase to create separation of the compounds for analysis. Reversed-phase liquid chromatography uses a non-polar stationary phase and a polar mobile phase, which is the opposite set of conditions from the classical mode of normal-phase liquid chromatography. The non-polar stationary phase, which usually consists of hydrocarbon chains bonded to an inert material, has a stronger affinity for less polar or hydrophobic compounds. Therefore, more polar or hydrophilic compounds will elute earlier in reversed-phase HPLC. The polar mobile phase usually consists of an aqueous blend of water with a miscible, polar organic solvent, such as acetonitrile or methanol. The most common reversed-phase HPLC columns, C₁₈ columns, use an octadecyl carbon chain (C₁₈)-bonded silica packing as the stationary phase.
7. **Describe another way to confirm aflatoxin B₁ or G₁ found in a sample other than by TFA derivative formation.** Confirmation of identity by liquid chromatography-mass spectrometry (LC-MS).
8. **How many grams of ground in-shell pistachios should be weighed to obtain 25 grams of nutmeat for analysis?** 50 grams should be weighed. Note: In-shell pistachios are 50% nut meat by weight. For different ratios, an equation can be set up such as: 0.50X = 25 g, where X = the amount of sample to be weighed.

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9. **What references would be consulted first for information on new or improved techniques for the determination of aflatoxins or other mycotoxins?** The Journal of the Association of Analytical Chemists, the Journal of Agricultural and Food Chemistry, Food Additives and Contaminants (found on the internet through Medline), or Science Direct are good sources.

6.3.2 Aflatoxin M₁

1. **What is the acceptable level of aflatoxin M₁ in milk?** Aflatoxin M₁ level in milk samples should be less than 0.5 ppb.
2. **What is the purpose of centrifugation at low temperatures?** Centrifugation at low temperatures allows the sample to completely precipitate thereby producing a clean extract.
3. **Why are sodium chloride (NaCl) and magnesium sulfate (MgSO₄) used in this method?** NaCl and MgSO₄ are used as drying agents to remove the water from the organic layer.
4. **In using the immunoaffinity method, are antibodies on the immunoaffinity column (IAC) monoclonal or polyclonal?** Monoclonal antibodies since they bind to a specific antigen (aflatoxin M₁).

6.3.3 Ochratoxin A

1. **What is an advantage of using immunoaffinity column method?** The immunoaffinity column uses a very selective antibody that binds only with ochratoxin A, which acts as the antigen. Other components of the matrix in the sample extract are washed from the column prior to the elution of ochratoxin A, therefore eliminating sources of potential interference or contamination.
2. **Why is a buffer solution of pH 7.4 needed for this method?** The antibody in the immunoaffinity column generally works best around a pH of 7. Any changes in pH will affect its binding capacity for capturing the antigen.

6.3.4 Zearalenone

1. **What is the method detection level (MDL) and limit of quantitation (LOQ) for this method?** The method detection level (MDL) is about 24 ng/g and the limit of quantitation (LOQ) is approximately 50 ng/g (about 2.1 times the MDL).
2. **What other mycotoxin can be separated using this method and how?** Aflatoxins B₁, B₂, G₁, and G₂ can also be separated using this method by using the same extraction procedure but separate immunoaffinity columns.

6.3.5 Trichothecenes

1. **Can conclusive identification be achieved by equivalent HPLC retention times (RT) between a sample peak and the DON standard peak alone? What**

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other method or instrument could be used for confirmation of identity of the compound found in the sample extract? No, it is possible for other compounds extracted from the sample to have the same retention time as DON. Confirmation of identity can be proven by comparing the compound in the sample extract with DON standard using mass spectrometry.

2. **Does DON have affinity to the SPE cleanup column packing used in J. AOAC Int. 79, 1996, 883-888 (see Section 5 References Y)?** No, DON passes through the charcoal/alumina/celite column. Interferences are mostly adsorbed on the column packing.
3. **Why is a combination of isocratic and step gradient conditions used in J. AOAC Int. 79, 1996, 883-888 (see Section 5 References Y)?** The isocratic condition is used to elute the DON peak. The use of a wash solvent or step gradient elution is to elute compounds after the elution of DON that may interfere with the next run and then re-equilibrate the column in the isocratic condition.
4. **Is DON stable during most processing procedures including baking?** Yes

6.3.6 Patulin

1. **Is there another way that the extraction could have been carried out? Discuss the advantages of the proposed way and the official method.** Separatory funnels instead of test tubes could be used to carry out the extraction. The advantage of this modified procedure is that it is easier to more completely separate the layers of solvents during the extraction without carrying over aqueous phase into the ethyl acetate as when using a disposable pipet. The advantage of the official method procedure is that it is easier to completely separate the layers of solvents without any loss of the ethyl acetate layer.
2. **What instruments can be used for determining the Brix value of a concentrate of apple juice?** A refractometer or a hydrometer is used for determining the Brix value of apple juice concentrate.
3. **Why is the combined ethyl acetate sample extract washed with 1.5% Na₂CO₃?** Sodium carbonate solution removes acidic interferences from the sample.
4. **What factors affect the stability of patulin standards and patulin sample extracts?** Heat and pH are factors that can affect patulin stability. Therefore, the method calls for diluting the standards and reconstituting the sample extracts with pH 4 water.
5. **What is the regulatory guidance for recommending legal actions against products collected for patulin analysis?** The FDA Compliance Policy Guide for patulin adulteration of apple juice, apple juice concentrates, and apple juice products gives the following criteria to be considered: The sample is analyzed in

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accordance with applicable methods of the AOAC, original and check analysis show patulin at or above 50 ppb in single strength juice, identity of patulin is confirmed by LC-MS.

6. **If the HPLC hydroxymethylfurfural (HMF) peak is close to the patulin peak, how may the separation between the two peaks be increased to obtain better resolution? What kinds of products are more likely to have a significant HMF peak present in the chromatogram?** If using acetonitrile, decrease the amount of acetonitrile in the mobile phase (i.e. 5% to 4%). If using water as the mobile phase, change to another column. From experience it has been found that processed apple juice products such as pasteurized apple juice, and especially apple juice concentrate tend to have more HMF. Fresh apple juice and sweet apple cider have little or no HMF.
7. **Why is it important to use anhydrous sodium sulfate to dry the ethyl acetate extract?** The main reason is given in AOAC method 995.10 which contains a note stating that patulin may be destroyed when wet ethyl acetate extract is evaporated to dryness. Anhydrous sodium sulfate removes water.

6.3.7 Fumonisin

1. **Why does the OPA reaction not work with fumonisins A₁ and A₂?** Fumonisin A₁ and A₂ are secondary amines.
2. **Why is there no screening procedure for fumonisins A₁ and A₂?** Fumonisin A₁ and A₂ are not as toxic as fumonisin B₁, B₂, and B₃.
3. **What condition occurs in horses upon ingesting high levels of fumonisin contaminated feed?** Horses develop a disease that causes a softening of the white matter in the brains. This disease is called equine leukoencephalomalacia (ELEM).
4. **What are the recommended maximum levels of fumonisins in human foods and animal feeds established by FDA?** The recommended maximum level of fumonisins in human foods is from 2-4 ppm based upon the particular corn-based product. The recommended maximum level of fumonisins in animal feed is from 1-100 ppm depending on the animals that the feed is intended for. (See Section 5 Reference A-B).

6.3.8 Multi-Mycotoxins Method

1. **What is the molecular weight of each native mycotoxin analyzed for in this method according to Section 5 Reference AA?** Aflatoxin B₁ = 312.1; Aflatoxin B₂ = 314.1; Aflatoxin G₁ = 328.1; Aflatoxin G₂ = 330.1; Deoxynivalenol = 296.1; Fumonisin B₁ = 721.4; Fumonisin B₂ = 705.4; Fumonisin B₃ = 705.4; HT-2 toxin = 424.2; Ochratoxin A = 403.1; T-2 toxin = 466.2; Zearalenone = 318.1

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2. **Explain how LC-MS/MS definitively confirms the identity of a substance and why, even though liquid chromatography on its own is non-specific, it becomes specific when paired with MS/MS.** Mass spectrometric specificity is based on the structure and abundance of ions or fragments. Ion abundance is, in turn, dependent on the structure and stability of the ions formed. Masses should match and ion abundances (or abundance ratios) should closely match those of a standard or a reference. Although chromatographic retention time is in itself non-specific, for identification the chromatographic retention time must match that of the compound whose mass spectrum is obtained.
3. **Describe how you would analyze a sample with a high level of one mycotoxin.** All violative sample findings for each analyte (potential Class 2 and Class 3) will be confirmed through check analysis by a second analyst. A check analysis requires the "Check" sample portion to be weighed out in triplicate, as well as one aliquot of the "Original" sample portion. Two of the "Check" aliquots will be spiked with the analyte of interest at a level near the amount found in the original analysis. All four aliquots will be run through either the multi-mycotoxins method or the individual method for the specific mycotoxin.