Contents

7.1 Introduction

Mycotoxins are natural poisons produced by fungi as secondary metabolites. Foods 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: the *Aspergillus sp*., *Penicillium sp*., and *Fusarium sp*. Of the numerous mycotoxins elaborated by these fungi, FDA is actively concerned with the aflatoxins, fumonisins, trichothecenes, ochratoxins, 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, inspection, 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 7.5 References 1-3). The compliance programs also list the analytical methods for each mycotoxin. For more information on mycotoxins, see Section 7.5 References 4-6. Chemical data (UV, IR, NMR, and mass spectra), general characteristics, and toxicity data may be found in Section 7.5 References 7.

A large amount of information about mycotoxins is found on the web. An example is found in Section 7.5 References 8, which covers the considerations for establishing an action level for patulin in apple juice, apple juice concentrate, and apple juice products. Other examples are found in Section 7.5 References 9, which is a background paper for fumonisin levels for corn and corn products intended for human foods, and Section 7.5 References 10, which is industry guidance for fumonisin levels in human foods and animal feeds. Information on fusarium toxins such as T-2 toxin, diacetoxyscirpenol (DAS), deoxynivalenol (DON), fumonisins, and others is found in Section 7.5 References 11. For physicochemical data for selected fusarium, toxins, see Section 7.5 References 12. (For each mycotoxin that has an actionable level, there is a Compliance Policy Guide (CPG). 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 (e.g. CPG for paulin, Section 7.5 References 13). More information on these and other mycotoxins may be found in Section 7.5 References 14 -17.

7.2 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 suspect carcinogens in humans, and are highly toxic. 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. Prior to their disposal, the solutions should be treated with at least one-tenth their volume of 5-6% sodium hypochlorite (household bleach) according to the laboratory's chemical hygiene plan (see Section 7.5 References 18). Swab accidental spills of toxin with 1 % NaOCl bleach, leave 10 minutes, and then add 5 % aqueous acetone. Rinse all glassware exposed to aflatoxins with methanol, add 1 % NaOCl solution, and after 2 hours add acetone to 5 % of the total volume. Allow a 30-minute reaction and wash thoroughly (see Section 7.5 References 19, Chapter 49, Subchapter 1, page 1).

Weighing and transferring mycotoxins in dry form should be avoided; they should be dissolved in a solvent. 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. Their concentrations should be determined spectrophotometrically. Containers of

mycotoxin standard solutions should be tightly capped and their weights may be recorded for future reference before wrapping them in foil and storing them in a freezer (see Section 7.5 References 19, Chapter 49, 971.22E). Keeping records of the weights of stored solutions will provide a means of determining if the solutions have 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.

7.3 Sample Preparation

The objective of sample preparation in trace analysis is to obtain a sample of convenient size (usually 5-100 g) whose composition accurately reflects the composition of the bulk sample from which it was taken (usually 2-25 kg). The nature of mycotoxin contamination makes this objective more difficult to attain than for most other types of trace analysis. Because the molds that produce mycotoxins do not grow uniformly on the substrate, contamination of the commodity is usually far from uniform (See Section 7.5 References 19, 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 using three types of equipment. Particle size reduction can be accomplished using Vertical Cutter/Mixers, such as Hobart VCM, Urschel Comitrol, and Robot Coupe VCM. Sample size reduction may be accomplished using a Jones divider (riffle). Mixing may be done with a planetary mixer or V-shell blender. All three steps are carried out for all samples prepared for mycotoxin analysis, but not necessarily in the order given, or with the particular equipment mentioned. In some cases, the grinding equipment combines the particle size reduction with the mixing step. In other cases, the steps have to be performed separately because the grinding operation does not thoroughly mix the sample composite. Chose the proper equipment for each sample; a properly ground and mixed sample is called a “composite.” Following compositing of regulatory samples, two one-quart portions of the composite should be placed in two containers – one for an “original” analysis, and one for a “check” analysis. A one-quart container may be saved for surveillance samples in case a follow-up compliance or regulatory sample is needed for a positive analytical finding. Check analyses are not performed on surveillance samples that are not large enough to support regulatory action as per the Investigations Operations Manual (IOM) Sample Schedule Chart Six or the compliance program. The remainder of the sample composite is disposed. In the case of a violative sample, the remaining original and check portions should be sealed and returned to the Sample Custodian for a reserve.

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 guidance on the preparation of nut samples, consult see Section 7.5 References 20-22. 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 not be homogeneous, 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. Note: *Aspergillus sp., penicillium sp.* will not grow under refrigeration or freezing, but *fusarium sp* will grow under refrigeration or freezing. The trainer will discuss the selection and proper use of the sample preparation equipment in the laboratory.

7.4 Exercises

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

The training analysts will instruct each trainee in the proper sample preparation procedure for each commodity, The training analysts should obtain bulk samples of 5-25 kg each of whole-kernel corn, shelled peanuts, and wheat or barley. Approximately 1 kg of each prepared commodity should be given to each trainee for use in the following exercises. The method numbers in the exercises refer to Official Methods of Analysis, (AOAC) Chapter 49, designated as Section 7.5 References 19.

Note: Training on *all* the various mycotoxin methods is *not* needed if the laboratory does not perform those analyses. Training on the TLC methods for aflatoxins is not mandatory if the laboratory does not use that technique. Even though not all of the quantitative methods use TLC, the official method for confirmation of identity of aflatoxin B_1 by LC/MS for most violative samples when MS instrumentation is available.

A. Questions

1. What is the difference between mycotoxin compliance and surveillance samples?
2. Using Sample Schedule, Chart Six, Mycotoxin Sample Sizes, IOM, (see Section 7.5 References 23) determine whether the following samples are surveillance or compliance samples:
    a. A 12 sub x 1 lb. sample of chunky peanut butter (=12 lb. sample)
    b. A 12 sub x 1 lb. sample of creamy peanut butter (=12 lb. sample)
    c. A 10 sub x 1 lb. sample of shelled almonds (=10 lb. sample)

d. A 50 sub x 1 lb. sample of shelled almonds (=50 lb. sample)

e. A 10 sub x 1.5 lb. sample of almond paste (=15 lb. sample)

f. A 10 sub x 1 lb. sample of corn (=10 lb. sample).

7.4.1 Aflatoxins B_1, B_2, G_1 and G_2

Aflatoxins are metabolic products of the molds, *Apergillus flavus* and *Apergillus 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, and watermelon seeds. Other nuts, grains, and seeds are susceptible but less prone to contamination with aflatoxins. Because aflatoxins are known carcinogens to laboratory animals and presumably man, 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, it is called a nutmeat basis. The units are µg/kg or ppb.

I. Thin Layer Chromatography

A. Assignments

1. Prepare standard solutions of aflatoxins B_1, B_2, G_1, and G_2 for thin layer chromatography (TLC) as described in AOAC 971.22.

2. Weigh four 50 g portions of the shelled peanut sample prepared earlier into 500 mL glass-stoppered Erlenmeyer flasks. Two of these samples are to be spiked with aflatoxins added at the same level by the trainer, and two will be analyzed as blanks. Analyze all four samples as described in AOAC 968.22. Identify and calculate the quantities of the aflatoxins found. Be prepared to discuss the results and any problems encountered. Reserve a second 50 mL portion of the extract from one of the blank samples. Evaporate the solvent over a steam bath and determine the volume of fat in the sample. Retain the final extracts for use in the next step.

3. Confirm aflatoxins found in the samples (step 2) by derivative formation as described in AOAC 975.37. Obtain and read Section 7.5 References 24-29.

B. Questions

1. What are aflatoxins? How do they affect humans and animals?

2. Why is the preparation of a homogeneous sample so critical in this determination?

3. How do the names of these four aflatoxins (B_1, B_2, G_1, and G_2) correlate with their appearance and chromatographic pattern on a TLC plate?

4. Several AOAC and Laboratory Information Bulletins (LIB) methods are used for the determination of aflatoxins in a number of commodities. Given the constraints on an analytical chemist working for a regulatory agency, which of the methods would one select to analyze a sample of peanuts for aflatoxins? For corn or for animal feed? Why?

5. What is the derivative that is prepared in the confirmation method in AOAC 975.37? What is the reaction? Which of these four aflatoxins can be derivatized using trifluoroacetic acid (TFA)? Why? How does this method differ from the derivative formed in AOAC 970.47? Which procedure would one use? Why?

6. Two samples of shelled filberts are received, one sample of in-shell pecans, and a sample of shelled walnuts. The samples are to be analyzed as described in AOAC 968.22. What details peculiar to each of these samples requires attention in order to perform an accurate analysis?

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

II. Liquid Chromatography

A. Assignments

1. Prepare standard solutions of aflatoxins B1, B2, G1, and G2 for high pressure liquid chromatography (HPLC) as described in AOAC 991.31D (g).

2. Weigh four 25 g portions of prepared shelled peanut in blender with cover. Two of these samples are to be spiked with aflatoxins added at the same level by the trainer, and two will be analyzed as blanks. Analyze all four samples as described in 991.31. Identify and calculate the quantities of the aflatoxins found. Be prepared to discuss the results and any problems encountered. Refer to Volume IV, Mycotoxin Analysis, Section 7.4.1 Part I-Thin Layer Chromatography, and No.3 (above) for confirmation of aflatoxins.

B. Questions

1. In what order do these four aflatoxins (B1, B2, G2, and G2) show up on the chromatogram using this method (reverse phase chromatography)?

2. Why is the dilution of the sample critical before placing the sample solution on the immunoaffinity column?

3. AOAC method 991.31 is for corn, raw peanuts, and peanut butter. Describe the methodology used for other commodities.

4. For AIAC method 991.31, what are levels of quantitation (LOQ) for aflatoxins B1, B2, G1, and G2? How does this compare with the LOQs using thin layer chromatography?

5. Explain the principles of reverse phase HPLC.

6. Describe another way to confirm aflatoxin B_1 or G_1 found in a sample by TFA derivative formation, other than by the TLC method specified in I. A No. 3 above.

7. How many grams of ground in-shell pistachios should be weighed to obtain 25 g of nutmeat for analysis?

8. Does the method found in Section 7.5 Reference 30 always give accurate results? Discuss.

9. (If not doing the TLC unit) Answer questions 1, 2, 4, 5, and 7 under Volume IV, Mycotoxin Analysis, Section 7.4.1, I. Thin Layer Chromatography, above.

7.4.2 Aflatoxin M_1

Aflatoxin M_1 is produced by lactating animals consuming aflatoxin contaminated feed. Aflatoxin B_1 is metabolized into aflatoxin M_1.

I. Thin Layer Chromatography

A. Assignments

1. Prepare aflatoxin M_1, standard solution as described in 980.21B (f).

2. Transfer four 50 mL aliquots of a milk sample obtained from the trainer into 250 mL Erlenmeyer flasks. Two of these samples are to be spiked by the trainer. Analyze all four samples as described in 980.21A-E.

3. Confirm the identity of the aflatoxin M_1, in one of the spiked samples as described in 980.21E.

B. Questions

What is the minimum detectable quantity (MDQ) of aflatoxin M_1, by this method? How does this compare with the MDQ for the other aflatoxins?

1. Is there a regulatory guideline for aflatoxin M_1, in milk? If so, what is it?

II. Liquid Chromatography

A. Assignments – SPE Method

1. Prepare aflatoxin M_1, standard solution as described in AOAC 986.16B (e).

2. Transfer four 20 mL aliquots of a milk sample obtained from the trainer into graduated cylinders containing 20 mL hot (ca 80 °C) water. Two of these samples are to be spiked by the trainer. Analyze all four samples as described in AOAC 986.16A-E.

Assignments – Immunoaffinity Method

1. Prepare aflatoxin M1 standard as described in AOAC 2000.08 D (c).

2. Transfer four 50 mL aliquots of a milk sample obtained from the trainer into graduate cylinders and warm in a water bath @ ca 37 C. Two of these samples are to be spiked by the trainer. Analyze all four samples as described in AOAC 2000.08 E-G.

C. Questions

1. What reaction occurs when aflatoxin M1 is derivatized using trifloroacetic acid (TFA)?

2. What is the purpose for using the C-18 cartridge and silica gel column in this method?

3. What is the principle of the procedure found in Section 7.5 References 31, and what are the advantages of using this method? Would this method, alone, be used for analyzing a violative sample?

4. In using the immunoaffinity method are antibodies on the immunoaffinity column (IAC) monoclonal or polyclonal?

7.4.3 Ochratoxin

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 is 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.

I. SPE Method, HPLC

A. Assignments

1. Prepare ochratoxin A standard stock solution of ca 24 µg/mL in toluene-acetic acid (99+1) as described in the European Committee for Standardization method (see

Section 7.5 References 32-33), a modification of AOAC 991.44C(f), determining the concentration spectrophotometrically. Dilute the stock solution to obtain a working standard solution of 4 µg/ml. Alternately, prepare 5 mL of a working standard solution of 0.4 µg/mL in toluene-acetic acid (99+1). This will result in HPLC injection standards of 1/10 the concentration described in the method. These injection standards will be closer in concentration to those of the immunoaffinity method.

2. Weigh four 50 g portions of wheat or barley into blenders with covers. Two of these samples are to be spiked with added ochratoxin A by the trainer. Analyze all four samples as described in (see Section 7.5 References 32).

3. Confirm the identity of the ochratoxin A found in one of the spiked samples as described in AOAC 991.44J or reference (see Section 7.5 References 32, 34).

B. Questions

1. The principle of separation of aflatoxins on silica gel is adsorption chromatography. What is the principle of separation of ochratoxin A in AOAC 991.44?

2. Using standard chemical structure notation, describe the reaction of ochratoxin A with BF_3 in methanol.

3. If ochratoxins are found in a sample, what other mycotoxins might be found?

II. Immunoaffinity Method, HPLC

A. Assignments

1. Prepare standard ochratoxin A to concentrations in (see Section 7.5 References 34). In this method, 5 mg of standard is weighed.

2. Weigh four 25 g portions of wheat or barley in blenders with covers. Two of the samples are to be spiked with added ochratoxin A by the trainer. Analyze all four samples by Section 7.5 References 34.

3. Confirm the identity of the ochratoxin A found in one of the spiked samples as described in the method.

B. Questions

1. What are the advantages of using the immunoaffinity method over the SPE method?

2. Why is a buffer solution of pH 7.4 needed for this method?

7.4.4 **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 found also in other important crops such as wheat, barley, sorghum, and rye.

A. Assignments

1. Prepare standard solution of zearalenone as described in AOAC 985.18C (a).
2. Weigh four 50 g portions into 500 mL glass-stoppered extraction flask. Two of these samples are to be spiked by the trainer. Analyze all four samples as described in AOAC 985.18A-G.
3. Confirm the identity of zearalenone found in both spiked samples as described in AOAC 985.18H.

NOTE: Solid phase extraction column (SPE) and immunoaffinity column (IAC) are alternative methods for zearalenone analysis. The following methods can be used:

- Ware, et al: Journal of AOAC International Volume 2, No. 1, 1999 (SPE column)
- McDonald, et al: Journal of AOAC International Volume 88, No. 6 2005 (IAC).

B. Questions

1. Describe the confirmation step in this method. How reliable is this confirmation method?
2. If zearalenone is found in a sample of wheat or corn, what other mycotoxins might be found? What other mycotoxins probably would not be found?

7.4.5 Trichothecenes

The trichothecene mycotoxins (i.e. T-2, DON) 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)

A. Assignments

1. Prepare fresh deoxynivalenol (DON) stock solution as described in AOAC 986.17 (see Section 7.5 References 27). Follow the journal method (see Section 7.5 References 27) to prepare a more dilute stock of 105 ug per 2100 uL of methanol-water (1+1). Finally prepare a 5 ng DON/uL working standard as per the method.

2. Weigh four 25 g portions of wheat or corn into 250 mL Erlenmeyer flasks or blenders with covers. Two of these samples are to be spiked with added DON by the trainer. Analyze all four samples for DON as described in (see Section 7.5 References 35).

NOTE: Immunoaffinity column (IAC) is an alternative method for deoxynivalenol analysis. The following method can be used:

- McDonald, et al: Journal of AOAC International Volume 86, No. 4 2005

B. Questions

1. Is the HPLC retention time (RT) of a chromatographic sample injection peak being equal to the DON standard peak's RT sufficient to provide conclusive identification? 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 cleanup column packing?

3. Why is a combination of isocratic and step gradient elution used in the HPLC?

4. Is DON stable during most processing procedures including baking?

7.4.6 Patulin

Patulin is a mycotoxin that is produced by certain species of *Penicillium, Apergillus,* and *Byssochylamys* 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.

A. Assignments

1. Prepare a standard stock solution of patulin for HPLC as described in AOAC 995.10C (e) calculating the patulin concentration as in AOAC 974.18C (d). Prepare patulin standard working solutions as in AOAC 995.10C (f) at the time of analysis.

2. Measure 5 mL apple juice or diluted concentrate into four 20 x 150 mm glass culture tubes. Two of these samples are to be spiked with patulin added at the same level by the trainer, and two will be analyzed as blanks. Analyze all four samples as described in AOAC 995.10. Identify and calculate the quantities of the patulin found. Be prepared to discuss the results and any problems encountered.

   Note: As per the CPs, samples of frozen concentrate or bulk concentrate should be diluted either as per recommendation for dilution or to a Brix value of 11.5° (single

strength) before analysis. See Section 7.5 References 19, Chapter 44, AOAC 932.14C, Solids in Syrup.

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 instrument is used for determining Brix value of a frozen concentrate of apple juice?

3. Why is the combined ethyl acetate sample extract washed with 1.5 % Na_2CO_3?

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 kind of products is 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?

7.4.7 Fumonisins

Fumonisins are natural toxins produced by *Fusarium moniliforme*, and other *Fusarium* species; these molds are common natural contaminants of corn. Fumonisins have been linked to fatalities in horses and swine. Recent studies have demonstrated the presence of fumonisins in human foods, including corn meal and breakfast cereals. More than ten types of fumonisins have been isolated and characterized. Of these, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) are the major fumonisins produced in nature.

A. Assignments

1. Prepare fresh fumonisin B_1, B_2, and B_3 standards by the AOAC 995.15 procedure.

2. Weigh four 50 g portions of ground corn into 250 mL plastic centrifuge bottles. Two of these samples are to be spiked with added fumonisin by the trainer. Analyze by above procedure.

B. Questions

1. Why does the OPA reaction not work with fumonisins A_1 and A_2?

2. Why is there no screening procedure for fumonisins A_1 and A_2?

3. What condition occurs in horses upon ingesting high levels of fumonisin contaminated feed?

4. What are the recommended maximum levels of fumonisins in human foods and in animal feeds established by FDA?

Note on the Preparation of Standard Mycotoxin Solutions for Training:

Mycotoxin standards are toxic, hazardous to handle, expensive, and distributed only in small quantities. The method exercises include the complete methods starting from the preparation of concentrated standard stock solutions. The trainers should use judgment in deciding the starting point for trainees. The beginning point could be pure standards, concentrated stock solutions, or intermediate stock solutions. It may be impractical to train large numbers of analysts starting from pure standards, which are toxic and should be handled by experienced analysts.

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7.6 Appendix – Answer Key

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 has to be collected and analyzed. Regulatory action may be taken on compliance sample results, not surveillance size samples.

2. **Using Sample Schedule, Chart 6, Mycotoxin Sample Sixes, IOM, (See Section 7.5 References, 23) determine whether the following samples are surveillance or compliance samples:**

    a. **A 12 sub x 1 lb. sample of chunky peanut butter (=12 lb. sample)** Surveillance sample

    b. **A 12 sub x 1 lb. sample of creamy peanut butter (=12 lb. sample**) Compliance sample

    c. **A 10 sub x 1 lb. sample of shelled almonds (=10 lb. sample**) Surveillance sample

    d. **A 50 sub x 1 lb. sample of shelled almonds (=50 lb. sample**) Compliance sample

    e. **A 10 sub x 1.5 lb. sample of almond paste (=15 lb. sample)** Compliance sample

    f. **A 10 sub x 1 lb. sample of corn (=10 lb. sample)** Compliance sample

7.4.1 Aflatoxins B_1, B_2, G_1 and G_2

I. Thin Layer Chromatography

1. **What are aflatoxins? How do they affect humans and animals**? Aflatoxins are a group of toxins produced by the molds, *Apersgillus flavus* and *Apergillus parasiticus* as metabolic products. The toxins consist primarily of aflatoxin B1, B2, G1, G2. M1 is produced from B1 in cow's milk. In animals, aflatoxins can cause liver cancer, decrease the production of milk and eggs, suppress the immune system, and are mutagenic. Aflatoxin B1 is the most potent carcinogen.

2. **Why is the preparation of a homogeneous sample so critical in this determination?** Both sampling and laboratory preparation of a homogeneous sample composite are important to obtain a 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 homogeneous but occurs in pockets of high contamination. Sample preparation results in a in a finely ground, evenly sized, particle sample composite.

3. **How do the names of these four aflatoxins (B_1, B_2, G_1, and G_2) correlate with their appearance and chromatographic pattern on a TLC plate?** The order in which they appear on the TLC plate are B1, B2, G1, and G2, with B1 having the largest R_f, traveling the furthest towards the solvent front. 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. **Several AOAC and Laboratory Information Bulletins (LIB) methods are used for determination of aflatoxins in a number of commodities. Given the constraints on an analytical chemist working for a regulatory agency, which of the methods would one select to analyze a sample of peanuts for aflatoxins? For corn or for animal feed? Why?** The compliance programs list applicable methods. a. Peanuts, corn, peanut butter—If a TLC method is used: AOAC 968.22A-F (CB Method) (49.2.08). If an HPLC method is used: AOAC 991.31 (49.2.18) (Aflatest immunoaffinity column cleanup using HPLC with post column iodine derivatization). b. Animal feed—Depending on the ingredient(s)—for corn or peanut product feeds-- If a TLC method is used: AOAC 968.22A-F. If an HPLC method is used AOAC 991.31. For cottonseed feeds 980.20 (49.2.19) using either TLC or HPLC. The above methods are official procedures for analyzing these products. Non-official validated methods may be used for screening but check analysis should preferably be done using an official method. AOAC Method 991.31 uses smaller amounts of solvents and doesn't use chlorinated solvents making this method safer and more economical than the other methods.

5. **What is the derivative that is prepared in the confirmation method in AOAC 975.37? What is the reaction? Which of these four aflatoxins can be derivatized using trifluoroacetic acid (TFA)? Why? How does this method differ from the derivative formed in AOAC 970.47? Which procedure would be used? Why?** Trifluoroacetic acid

(TFA) is the derivatizing agent used for confirmation in AOAC 975.37. The reaction is based upon the catalytic action of TFA causing the addition of water across the double bond of the terminal furan ring of aflatoxin B1 and G1. Aflatoxin B1 and G1 can be derivatized using trifluoroacetic acid because both have a double bond in the furan ring structure. Aflatoxins B2 and G2 do not have this double bond.

The derivatives in AOAC 970.47 (a method found in AOAC 14th Ed., no longer current) are also formed in B1 and G1 at the same double bond site but with different reagents. An important difference is that the older method calls for purification of aflatoxin in the sample extract by preparatory TLC. The derivatization is carried out in glass vials with heat, and the resulting derivatives are identified by TLC. The newer method AOAC 975.37 is more direct and efficient, allowing formation of the derivatives directly on the TLC plate without requiring additional cleanup of the sample extract.

6. **Two samples of shelled filberts are received, one sample of in-shell pecans, and a sample of shelled walnuts. The samples are to be analyzed as described in AOAC 968.22. What details peculiar to each of these samples requires attention in order to perform an accurate analysis**? The amount of oil contained in the 50 mL filtered chloroform aliquot of sample extract for each product (fiberts, pecans, & walnuts) is to be determined. The ratio of nutmeat to whole product of in-shell tree nuts to be determined to calculate the aflatoxin level based on the nutmeat basis (assumes that all of the aflatoxin is contained in the edible kernels and none is in the shells). The per cent nutmeat or per cent kernels for some seeds are given in the mycotoxin compliance programs. The value of the oil for peanuts and peanut butter is given in the method as 5 g, resulting in an oil-corrected aliquot of 45 mL of chloroform being removed for column cleanup, with the final sample extract representing 9 g of sample test portion instead of 10 g.

7. **What reference would be consulted first for information on new or improved techniques for the determination of aflatoxins? For other mycotoxins?** The *Journal of the Association of Analytical Chemists* or *Food Additives and Contaminants* (found on the internet through Medline) are good sources. Science Direct on the internet is a good site.

II. Liquid Chromatography

1. **In what order do these four aflatoxins (B1, B2, G1, and G2) show up on the chromatogram using this method (reverse phase chromatrography)?** The four aflatoxins appear in the following order: G2, G1, B2, and B1.

2. **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.

3. **AOAC method 991.31 is for corn, raw peanuts and peanut butter. Describe the methodology used for other commodities.** Run a spike recovery and a blank of the same commodity. Generally, the recovery should be greater than 80 %. The chromatogram should show no interference in the area of the aflatoxin peaks.

4. **For AOAC method 991.31, what are levels of quantitation (LOQ) of aflatoxins B1, B2, G1? How does this compare with the LOQs using thin layer chromatography?** The level quantitation (LOQ) of aflatoxins B1, B2, G1, and G2 are 1.0 ng/g (ppb). The level of quantitation for the CB method (AOAC 49.2.08) is 1 to 6 ng/g (ppb) depending on the edible to whole product ratio, the amount of oil present in the sample, the dilution volume of the final sample extract, and whether it is B1, B2, G1, or G2 aflatoxin. The HPLC method is much more sensitive than the CB method.

5. **Explain the principles of reverse phase HPLC.** Reverse phase refers to a nonpolar stationary phase and a nonpolar mobile phase. Reversed phase chromatography utilizes the solubility properties of the sample in very much the same way as the organic chemist does when he purifies a crude sample by partitioning it between a hydrophilic and a lipophilic solvent in a separatory funnel. The partition of the sample components between the two phases will depend on their respective solubility characteristics. Less hydrophobic components will end up primarily in the hydrophilic phase while more hydrophobic ones will be found in the lipophilic phase. In a way, one can say that the whole process depends on the extractive power of the hydrophilic phase. This can be affected by the addition of an organic solvent which is soluble in the hydrophilic phase. A high concentration of the organic solvent will increase the extracting power for hydrophobic compounds.

   In reversed phase chromatography, silica particles covered with chemically bonded hydrocarbon chains represent the lipophilic phase (C2 to phenyl C18); while an aqueous mixture of an organic solvent, surrounding the particle represents the hydrophilic phase. When a sample component passes through a reverse phase chromatography column the partitioning mechanism operates continuously. Depending on the extractive power the hydrophilic phase (or the "eluant"), a greater or lesser part of the sample component will be retained reversibly by the lipid layer of the particles (the "stationary phase"). The larger the fraction retained in the lipid layer, the slower the sample component will move down the column. Hydrophilic compounds will always move faster than hydrophobic ones, since the mobile phase is always more hydrophilic than the stationary phase. Reference: http://ntri.tamuk.edu/fplc/rev.html

6. **Describe the ways to confirm aflatoxin B_1 or G_1 found in a sample.** TLC using TFA derivative formation and negative ion chemical ionization mass spectrometry are ways to confirm aflatoxin B1 and G1.

7. **How many grams of ground in-shell pistachios should be weighed to obtain 25 grams of nutmeat for analysis?** Note: Pistachios are 50% nut meat. The answer is 50 g. For

different ratios, an equation can be set up such as: 0.50X = 25 g, where X = the amount of sample to be weighed.

8. **Does the method found in Section 7.5 Reference 30 always give accurate results? Discuss**. No. The sugar in samples having high sugar content may interfere with bonding sites on the Aflatest column. In addition chloroform may be a better extracting solvent than methanol-water (7+3). AOAC 49.2.08 (CB method) is recommended to provide the analyst with more accurate quantitation of the aflatoxin present. The HPLC immunoaffinity column method may be used if when significant levels of aflatoxin are found the CB method or the CB method extraction is used to obtain a more accurate result.

9. **(If not doing the TLC unit) Answer questions 1, 2, 4, 5, and 7 under 7.4.1, I. Thin Layer Chromatography, above.** (See the answers above.)

7.4.2 Aflatoxin M_1

I. Thin Layer Chromatography

1. **What is the minimum detectable quantity (MDQ) of aflatoxin M_1, by this method? How does this compare with the MDQ for the other aflatoxins?** Based upon the diary product to be analyzed the MDQ ranges from 0.3 ng/g for cheese to 1.0 ng/g for powdered milk. This method is not as sensitive as HPLC methods. JAOAC Intl. Vol. 84, No. 2, 2001 is applicable for the determination of aflatoxin M_1 in raw liquid milk at>0.02 ng/mL. A greater sensitivity is needed for M1 methods because the guideline for M_1 aflatoxin in milk is much lower than for the other aflatoxins.

2. **Is there a regulatory guideline for aflatoxin M_1, in milk? If so, what is it?** Yes. According to Compliance Policy Guide 7106.10, legal action can be recommended if: the original and check analysis show that the sample contains greater than 0.5 ppb aflatoxin M_1 and the identity of aflatoxin M_1 is confirmed by chemical derivative test.

II. Liquid Chromatography

1. **What reaction occurs when aflatoxin M_1 is derivatized using trifloroacetic acid (TFA)?** The addition of water across the double bond of the terminal furan ring of aflatoxin M_1 occurs in this reaction due to the catalytic action of TFA.

2. **What is the purpose for using the C-18 cartridge and silica gel column in this method?** The C-18 cartridge replaces the liquid-liquid partitioning in the older methods in which chloroform was used. The silica gel is used to fractionate the sample extract based on polarity.

3. **What is the principle of the procedure found in Section 7.5 Reference 31, and what are the advantages of using this method? Would this method, alone, be used for analyzing a**

violative sample? The method uses an affinity column containing monoclonal antibodies sensitive for aflatoxin M_1. Aflatoxin M_1 binds to the antibody sites on the column and interfering compounds are eluted off with water. Aflatoxin M_1 is then removed from the antibodies using methanol. This method is fast, and calls for moderate amounts of methanol and distilled water compared to the solvents used for other methods. No. This method should not be used alone for analyzing violative samples. This method should be used for screening samples for aflatoxin M_1. An official method should be used for violative samples.

4. **In using the immunoaffinity method are antibodies on the immunoaffinity column (IAC) monoclonal or polyclonal?** Monoclonal antibody. Monoclonal antibodies are which binds to a specific antigen (aflatoxin M1).

7.4.3 Ochratoxins

I. SPE Method, HPLC

1. **The principle of separation of aflatoxins on silica gel is adsorption chromatography. What is the principle of separation of ochratoxin A in AOAC 991.44?** Reversed phase chromatography using a C18 column is the principle of separation of ochratoxin A using method AOAC 991.44.

2. **Using standard chemical notation, describe the reaction of ochratoxin A with BF_3 in methanol.**

$$RCOOH + MeOH + BF_3 \text{ -----> } RCOOCH_3$$

3. **If ochratoxins are found in a sample, what other mycotoxins also might be found?** Citrinin often occurs with ochratoxin A in cereal grains such as wheat, barley, oats, rice, and corn. Most fungi that produce citrinin also produce ochratoxin A.

II. Immunoaffinity Method, HPLC

1. **What are the advantages of using the immunoaffinity method over the SPE method?** The immunoaffinity column uses a very selective antibody that binds only with ochratoxin A (acts as the antigen) contained in the sample extract. Other components of the matrix are removed from the column with water. Small amounts of sample extract and solvents are needed in this method. The SPE method calls for chloroform, which this method does not.

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.

7.4.4 Zearalenone

1. **Describe the confirmation step in this method? How reliable is this confirmation**

method? The fluorescence measured at two excitation wavelengths is used for identification. Using the ratio of emission at two wavelengths is a more specific means of confirming the identity since it is less likely that an interfering compound would have the same emission characteristics as the zearalenone at two different excitation wavelengths. A mass spectral identification would be more specific and certain.

2. **If zearalenone is found in a sample of wheat or corn, what other mycotoxins might be found? What other mycotoxins probably would not be found?** *Fusarium graminearum* may produce deoxynivalenol along with zearalenone in wheat and corn. Aflatoxins would probably not be found since *Fusarium species* grow in moist cool conditions and aflatoxin is produced at warmer temperatures by *Aspergillus flavus* and usually in corn but not in wheat.

7.4.5 Trichothecenes [Deoxynivalenol (DON), a.k.a. Vomitoxin)

1. **Is the HPLC retention time (RT) of a chromatographic sample injection peak being equal to the DON standard peak's RT sufficient to provide conclusive identification? What 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 cleanup column packing?** 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 elutions used in the HPLC?** The isocratic elution is used to elute the DON peak while the step gradient elution is used to elute late eluters that may interfere with the next run.

4. **Is DON stable during most processing procedures including baking?** Yes.

7.4.6 *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.

2. **What instrument is used for determining Brix value of a frozen concentrate of apple juice?** A refractometer is used for determining the Brix value of apple juice concentrate.

3. **Why is the combined ethyl acetate sample extract washed with 1.5 % Na_2CO_3?** 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 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 guidance: The following criteria should be considered: The sample is analyzed in accordance with applicable methods of the AOAC, original and check analysis show patulin at or above 50 ppb on single strength juice, identity of patulin is confirmed by GC/MS.

6. **If the HPLC hydroxymethylfurfural (HMF) peak were close to the patulin peak, how may the separation between the two peaks be increased to obtain better resolution? What kind of products is 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.

7.4.7 *Fumonisins*

1. **Why does the OPA reaction not work with fumonisins A_1 and A_2?** Fumonisin A_1 and A_2 are secondary amines.

2. **Why is there non screening procedure for fumonisins A_1 and A_2?** Fumonisin A1 and A2 are not as toxic as fumonisin B_1, B_2, and B_3.

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.

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 5-100 ppm

depending on the animals that the feed is intended for. (see Section 7.5 Reference 10).

7.7 Document Change History

Version 1.3	Revision	Approved: 09-01-05	Author: LMEB	Approver: LMEB
Version 1.4	Revision	Approved: 02-02-10	Author: LMEB	Approver: LMEB
Version 1.5	Revision	Approved: 02-06-12	Author: LMEB	Approver: LMEB

Version 1.3 changes:
Table of Contents – 7.6 title change; 7.7 added.
Section 7.2 revised first sentence; 7.4.3 revised last sentence.

Version 1.4 changes:
NOTE: - revised last sentence
7.4.2 Aflatoxin M1 II. A. - revised
7.4.2 Aflatoxin M1 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 M1 – added 4.
7.6 7.4.7 – deleted 1.
Footer – updated web link

Version 1.5 changes:
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.