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).