Procedure for Aflatoxin M_1 and B_1 in Liver by HPLC-Fluorescence Detection with Pre-column Derivatization

1.0 PURPOSE

1.1 Determination of aflatoxin M_1 (AFM_1) and B_1 (AFB_1) in liver by HPLC-fluorescence detection with pre-column derivatization.

2.0 VALIDATION HISTORY

2.1 "In-house" validation data collected by 06/04/2014.

2.2 Level Three inter-laboratory validation: pending.

3.0 SAFETY PRECAUTIONS

3.1 Wear gloves, lab coat and protective eye wear. Conduct all steps involving organic compounds in fume hoods.

3.2 Aflatoxin M_1 and B_1 are toxic and carcinogenic and hence should be handled with extreme care.

4.0 CHEMICALS AND MATERIALS

4.1 The reference standard of aflatoxin M_1 and B_1, Sigma-Aldrich (St-Louis, MO, USA). Part #: aflatoxin M_1, A-6428; aflatoxin B_1, A-6636.

4.2 Acetonitrile, HPLC grade, Fisher Scientific (Waltham, MA, USA), Part #: A298-4.

4.3 Methanol, HPLC grade, Fisher Scientific (Waltham, MA, USA), Part #: A452-4.

4.4 Dichloromethane, pesticide grade, Fisher Scientific (Waltham, MA, USA), Part #: D142-4.

4.5 Trifluoroacetic acid (TFA), bioanalysis grade, Acros Organics, Fisher Scientific (Waltham, MA, USA), Code: 293811000.

4.6 Glacial acetic acid, A.C.S. reagent grade, Fisher Scientific (Waltham, MA, USA), Part #: A38c-212.

4.7 Celite 503, J.T. Baker. (now Avantor Performance Materials, Center Valley, PA, USA), Part #: E406-08.

4.8 Citric acid monohydrate, certified A.C.S. reagent grade, Fisher Scientific (Waltham, MA, USA), Part #: A104-500.

4.9 Sodium sulfate anhydrous, certified A.C.S. reagent grade, Fisher Scientific (Waltham, MA, USA), Part #: S421-3.

4.10 Octadecyl (C18) 40 µm Preparation LC packing, J. T. Baker (now Avantor Performance Materials, Center Valley, PA, USA.), Part #: 7025-01.

4.11 Alumina neutral, 60-325 mesh, for chromatography, Fisher Scientific (Waltham, MA, USA), Part #: A950-500.

4.12 All aqueous solutions are prepared in18.2 MΩ·cm water (deionized water) by Aries High Purity Water System (Aries Filter Network, USA) or equivalent.

4.13 Whatman@ 740-E filter discs, Sigma-Aldrich (St-Louis, MO, USA). Part #: 10328170

4.14 Glass fiber filter circles (9.0 cm), Fisher Scientific (Waltham, MA, USA), Cat. #: 09-804-90A.

4.15 7.0 mL borosilicate glass scintillation vials, Fisher Scientific (Waltham, MA, USA), Cat. #: 03-337-26.

4.16 Syringes, 6 mL, Monoject, Kendall, Part #: 1180600555.

4.17 55 mL screw cap glass tubes, Fisher Scientific (Waltham, MA, USA), Cat. #: 14-933D ***or 50 mL screw cap glass tubes***, Fisher Scientific (Waltham, MA, USA), Cat. #: 05-558-5B.

5.0 PREPARATION OF REAGENTS AND STANDARDS

5.1 The standard stock solutions of each aflatoxin is prepared by dissolving the pre-weighed standards in chloroform (AFM_1) or methanol (AFB_1) and stored in -20°C when not in use.

5.1.1 Example: A 5 µg AFM_1 standard is dissolved in 5 mL of chloroform to make a 1 µg/mL stock standard.

5.1.2 Example: A 5 mg AFB_1 standard is dissolved in 5 mL of methanol to make a 1 mg/mL stock standard.

5.2 A mixed standard solution (250 ng/mL for each aflatoxin) is prepared. Quantitatively transfer 1 mL of the AFM_1 stock standard and 1 µL of the AFB_1 stock standard to a 7 mL scintillation glass vial. Concentrate to dryness by nitrogen effusion and dilute in 4 mL methanol. It is stored at -20°C when not in use and is good for one year.

5.3 A working standard solution (25 ng/mL) is prepared by dilution of the 250 ng/mL standard solution using methanol. It is prepared on the day of use. Transfer 0.5 mL of the 250 ng/mL mixed standard to a 7 mL vial, add 4.5 mL of methanol and vortex to mix.

5.4 20% Citric acid solution is prepared by weighing 20.0 g of citric acid monohydrate and dissolving in de-ionized water to give a final volume of 100 mL. This amount is good for about 80-90 samples.

5.5 Acetonitrile/water 90/10 (v/v) is prepared by adding 100.0 mL de-ionized water to 900 mL acetonitrile and mixed well. This amount is good for 80-90 samples.

5.6 Octadecyl (C18) LC packing / alumina neutral 50/50 (w/w) is prepared by mixing 70.0 g alumina neutral and 70.0 g Octadecyl (C18) LC packing in a sealable plastic container. The mixture is shaken for five minutes. Shake for five seconds before each use. This amount is

good for about 80-90 samples.

5.7 Clean-up columns are prepared by weighing 1.50 g premixed 50/50 (w/w) Octadecyl (C18) 40 µm Preparation LC packing/Alumina Neutral into 6 mL plastic syringes. The C18/alumina mixture is immobilized by 2 Whatman 740-E filter discs on both ends. ***Filter discs are placed with the larger side facing up, and assure that the filter discs and the packing material are firmly seated in the syringe.***

5.8 Derivatization reagent 35/10/5 (v/v) water/TFA/glacial acetic acid: mix 10 mL TFA with 5 mL glacial acetic acid and 35 mL de-ionized water. Store in dark or aluminum-foil-wrapped bottles. This amount is good for approximately 100 derivatizations. This amount is good for about 100-120 samples. We suggest this is good for 3 months.

6.0 EQUIPMENT

6.1 Centrifuges. Bench top centrifuge (IEC Centra-GP8 or equivalent).

6.2 Balances. Capable of weighing minimum: 0.01 g (Mettler Toledo or equivalent).

6.3 Mixers and shakers. Single tube vortex mixer (VWR or equivalent), Rotor rack shaker (LabQuake, Thermo Scientific or equivalent).

6.4 Pipettes. Variable pipettes to cover ranges of 1.00-10.00 µL, 10.0-100.0 µL, 100-1000 µL, and 1-10 mL. (Eppendorf or equivalent).

6.5 Heating bath. Digital dry bath (GeneMate or equivalent).

6.6 A Waters high performance liquid chromatography equipped with a Waters 2695 separation module including a vacuum degasser, a quaternary pump, an automatic sample injection system, a Waters 2475 Multi-wavelength fluorescence detector, and the Empower software to control the instrument, data acquisition, and data analysis is used for separation and quantification of aflatoxins (or equivalent).

6.7 HPLC column. C18 reverse phase column (Brownlee C18, 5 µm, 80 Å, 100 mm × 4.6 mm, I.D. Perkin Elmer, Waltham, MA, USA, part #: 0711-0015). A guard column (Agilent Pursuit XRs C18, MetaGuard, 3µmm, 2.0 mm, part #: A6001MG2) is used.

7.0 SAMPLE PREPARATION

7.1 Preparation of calibration curve in liver matrix:

7.1.1 Seven approximately 1.0 g control liver samples are weighed in 50 mL screw cap glass tubes.

7.1.2 A series of volumes (8.0 µL, 20.0 µL, 40.0 µL, 80.0 µL, 200 µL, and 400 µL) of the 25 ng/mL working standard solution of aflatoxin M_1 and B_1 is spiked to 1.0 g of

control liver to give a series of fortified concentration of 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 ng/g.

7.1.3 The fortified samples are mixed thoroughly by vortexing for 10 sec at maximum speed and subject to the following steps 7.2-7.4.

7.2 Extraction: [1]

7.2.1 Approximately 1.0 g of liver samples are weighed in 50 mL screw cap glass tubes.

7.2.2 1.0 mL of 20% citric acid solution is added to each sample and the samples are subsequently mixed thoroughly by vortexing for approximately 10 sec at maximum speed. Samples are allowed to sit for 5-10 minutes and mixed again.

7.2.3 0.20 g Celite™ is added to each sample and followed by vortexing for 10 sec at maximum speed. If the initial mass of liver is not 1.0 g, then the amount of Celite is adjusted accordingly based on the ratio 1.0:0.2 (liver mass: Celite, w/w).

7.2.4 20.0 mL of dichloromethane is then added to each sample and the mixtures are vortexed for 10 seconds.

7.2.5 The mixture is mounted on Roto rack for rotate-mixing for 35 minutes, followed by centrifuge at 2000 rpm for 10 minutes.

7.2.6 After centrifugation, the bottom clear layer is carefully aspirated and passed through sodium sulfate (7.20 g) housed in a set of glass fiber filter circle and plastic funnel. The filtrates are collected in clean 50 mL tubes.

7.2.7 Sodium sulfate is then washed with 6 mL dichloromethane. The wash is combined with the solution obtained from 7.2.6.

7.2.8 The solutions are subsequently concentrated to dryness by gentle nitrogen effusion at ambient temperature.

7.3 Clean-up:

7.3.1 The dried extracts obtained in the previous step are reconstituted in 5.0 mL 90/10 (v/v) acetonitrile/water and vortexed for 10 sec at maximum speed.

7.3.2 Clean-up columns are pre-conditioned by 5.0 mL 90/10 (v/v) acetonitrile/water before use. ***The acetonitrile/water and vials are discarded.***

7.3.3 The dissolved extracts are then loaded on clean-up columns. The solution flowing through the columns is collected in a ***new*** 7.0 mL scintillation vial. Solutions are allowed to flow through by gravity. After all the solution flows through the column, the plungers are pushed down to the barrel of the syringe to drive out the residual

liquid in the packing. The residual liquid is received in the same 7.0 mL vials for each sample.

7.3.4 The solutions are subsequently concentrated to dryness by gentle nitrogen effusion at ambient temperature.

7.4 Derivatization:

7.4.1 The residue obtained in the previous step are reconstituted in 400 µL 35/10/5 (v/v) water/TFA/glacial acetic acid, vortexed for 10 sec at maximum speed, then heated at 65°C in heating dry bath for 15 minutes.[2]

7.4.2 The solutions obtained from this step are incubated at ambient temperature for 16-20 hours ***(This incubation time needs to be optimized under each lab's conditions, see Appendix)*** before HPLC analysis.

8.0 HPLC CONDITIONS

8.1 The optimized excitation and emission wavelengths for the fluorescence detector are 360 and 440 nm, respectively.

8.2 The mobile phase consisting of water (A) and acetonitrile (B) is pumped at a flow rate of 1.0 mL/min. A gradient elution is used to give the optimized separation. The details of the gradient program is as follows: 0-7.5 min isocratic step at 86% A, 14% B; 0.5 min linear gradient (7.5-8.0 min) to 85% A, 15% B, an isocratic step from 8.0 to 20.0 min at 85% A, 15% B; a 1.0 min linear gradient (20.0-21.0 min) to 86% A, 14% B; and a final isocratic step at 86% A, 14% B to the end. A total running time is 25 min. ***(This needs to be optimized according to each lab's HPLC system, see Appendix).***

8.3 An injection volume of 20 µL is used.

8.4 Retention time: based on the above-mentioned HPLC conditions, aflatoxin M_1 chromatographs at approximately 4.8 min, aflatoxin B_1 chromatographs at approximately 18.2 min.

9.0 RESULT INTERPRETATION

9.1 The calibration curve is created by plotting the blank-subtracted fluorescence intensity (the peak area of fluorescence unit) versus the injected mass of standard (in ng) by linear regression.

10.0 REFERENCES

10.1 AOAC Official Methods 982.24 Aflatoxin B_1 and M_1 in liver. Thin-Layer Chromatographic Method. AOAC INTERNATIONAL Gaithersburg, MD, USA.

10.2 AOAC Official Methods 994.08 Aflatoxin in Corn, Almonds, Brazil Nuts, Peanuts, and Pistachio Nuts. June 2000, AOAC INTERNATIONAL Gaithersburg, MD, USA.

11.0 APPENDIX

11.1 Each lab should perform a practice run to check the optimal incubation time and HPLC run time at three levels (low at 0.8 ppb, medium at 5 ppb, and high at 8 ppb). After incubation, inject samples at 20 h, 24 h, 28 h, and 32 h to check signal drift for each aflatoxin. The optimal incubation time is the time when minimal signal drift is observed. A signal drift of ± 15% is acceptable.

11.2 The HPLC run time is optimal as long as the last eluting peak has returned to the baseline (2-4 minutes) before the run has ended. The validated run time is 25 minutes, if needed the run time can be extended in 5-10 minutes increments until the run time has been optimized.

Note, this method was published:

1. Intralaboratory development and evaluation of a high-performance liquid chromatography-fluorescence method for detection and quantitation of aflatoxins M1, B1, B2, G1, and G2 in animal liver. Dahai Shao 1, Paula M Imerman 1, Dwayne E Schrunk 1, Steve M Ensley 1, Wilson K Rumbeiha. J Vet Diagn Invest. 2016 Nov;28(6):646-655. doi: 10.1177/1040638716668217. Epub 2016 Sep 16. https://pubmed.ncbi.nlm.nih.gov/27638844/

2. Evaluation of a Diagnostic Method to Quantify Aflatoxins B1 and M1 in Animal Liver by High-Performance Liquid Chromatography with Fluorescence Detection. Xiangwei Du, Dwayne E Schrunk, Paula M Imerman, Lori Smith, Kyle Francis, John Tahara, Andriy Tkachenko, Renate Reimschuessel, Wilson K Rumbeiha. J AOAC Int. 2019 Sep 1;102(5):1530-1534. doi: 10.5740/jaoacint.18-0355. Epub 2019 Feb 8. https://pubmed.ncbi.nlm.nih.gov/30736868/