New Jersey State Department of Agriculture

Assessing Presence of Aflatoxins and Ochratoxin A in Medical Marijuana Using HPLC

Effective Date: 04/30/2019 **Revision # 1**

1. Introduction

Mycotoxins are a group of naturally occurring toxic chemicals produced as secondary metabolites by fungi[1]. Their presence on agriculture commodities and related food products have been shown to cause a variety of adverse health effects in humans, including cancer and liver damage[2].

The FDA regulates mycotoxins with an authority established by the Federal Food, Drug and Cosmetic Act (FFDCA). Of the numerous mycotoxins produced by fungi, the FDA is most concerned with the aflatoxins B1, B2, G1, G2 and M1; Ochratoxin A; fumonisins B1, B2 and B3; trichothecenes (principally nivalenol, deoxynivalenol, T-2 and HT-2 toxin), patulin, and zearalenone. Specifically, FDA has promulgated regulatory guidance for three mycotoxins: action levels for aflatoxins and advisory levels for deoxynivalenol (vomitoxin) and fumonisins. For example, FDA set up a limit of 20 ppb of aflatoxins (B1+B2+G1+G2) for human food and animal feeds (corn and other grains) intended for immature animals or unknown destinations[3].

The New Jersey Department of Agriculture (NJDA) cooperates with the New Jersey Department of Health to perform aflatoxins (B1, B2, G1, and G2) and Ochratoxin A analyses on medical marijuana. The preferred quantitative method for these mycotoxins determination is liquid chromatogram (LC) with mass spectrometry (MS) detection. High performance liquid chromatography (HPLC) is an acceptable alternative method to LC/MS, and is what NJDA currently uses.

Two method difficulties prevent a regular HPLC method, i.e. a HPLC with ultraviolet (UV) and visible light detection for Aflatoxins and Ochratoxin A, from being implemented. The Aflatoxins molar absorptivity is so small that either a long path detection cell is needed or a high level of aflatoxins must be present in sample to produce substantial response for detection and quantification[4]. Therefore, HPLC with UV detection cannot be used for trace level aflatoxins analyses. To overcome this difficulty, a HPLC with fluorescence detection was decided to analyze aflatoxins as well as ochratoxin A. Fluorescence detection is far more sensitive than UV absorption and can detect trace levels of aflatoxins. To further increase the fluorescence detection sensitivity, a photochemical derivatization after HPLC column separation is applied.

The determination of mycotoxins requires a cleanup of plant extractions prior to being injected to HPLC instrument. This method uses a single immunoaffinity column for the purification. Only Aflatoxins (G2, G1, B2, and B1) and Ochratoxin A can be retained on the immunoaffinity column and then eluted, achieving a separation of target compounds from interfering chemicals.

2. Purpose

This standard operating procedure (SOP) provides instructions to be followed to determine Ochratoxin A and Aflatoxins (B1, B2, G1, and G2) in extraction of marijuana plants using HPLC with fluorescence detector conjunction with photochemical reaction device.

3. Safety

3.1. Always wear appropriate PPE (safety googles/glasses, lab coat and gloves).

3.2. Always add acid to water and not vice versa. Acid may otherwise splash out of a container and/or onto personnel. Make sure to work under a Chemical fume hood when working with acid solutions.

3.3. Exercise caution when measuring out liquid quantities with the glass syringes.

4. Materials

4.1. Reagents

4.1.1. Milli-Q Water or equivalent
4.1.2. Acetonitrile, HPLC grade or equivalent
4.1.3. Methanol, HPLC grade or equivalent
4.1.4. Phosphoric Acid, HPLC grade or equivalent
4.1.5. PBST Powder
4.1.6. Sodium Bicarbonate ($NaHCO_3$)

4.2. Supplies

4.2.1. 30mL syringes
4.2.2. 10mL syringes
4.2.3. Glass wool
4.2.4. 10mL centrifuge tubes
4.2.5. AflaOchra HPLC Immunoaffinity column
4.2.6. 2mL amber vials with screw-on caps
4.2.7. 5mL Volumetric Flasks
4.2.8. Hamilton 10µL syringe, point style 3
4.2.9. Hamilton 25µL syringe, point style 3
4.2.10. Hamilton 100µL syringe, point style 3
4.2.11. Hamilton 500µL syringe, point style 3
4.2.12. Disposable pipets
4.2.13. Aflatoxins Mix Standard (2600ppb) or equivalent
4.2.14. Ochratoxin A Standard (50,000ppb) or equivalent
4.2.15. Agilent Zorbax Eclipse XDB-C18, 150 mm × 4.6 mm (I.D.), 3.5 µm particle size (Part # 963967-902) or equivalent

4.3. Equipment

4.3.1. Analytical Balance – capable of weighing 0.001 g
4.3.2. Centrifuge or equivalent
4.3.3. Vortex mixer
4.3.4. Agilent 1200 Series HPLC consisting:
Agilent 1200 Series Degasser (G1379B)
Agilent 1200 Series Binary Pump (G1312A)
Agilent 1200 Series Autosampler (G1329A)
Agilent 1200 Series Thermostatted Column Compartment (G1316A),
Agilent 1200 Series Fluorescence Detector (G1321A)
Agilent 1200 Series Diode Array and Multiple Wavelength Detector (G1315B)
4.3.5. Photochemical reactor (PhCR) or equivalent

5. Procedure

5.1. Solution Preparation

5.1.1. PBST Buffer Solution (1%, w/v):
Weigh and dissolve 5.0g of PBST powder into 500mL Milli-Q water. Check that the pH of the buffer solution is between 7.2 and 7.6.

5.1.2. Sodium Bicarbonate ($NaHCO_3$) Solution (0.5%, w/v):
Weigh and dissolve 5.0g of Sodium Bicarbonate into 1000mL of Milli-Q water. Check that the pH of the solution is between 8.4 and 8.6.

5.1.3. Extraction Solution:

Prepare a mixture of methanol and 0.5% Sodium Bicarbonate solution in the ratio of 70:30 (v/v).

5.1.4. Immunoaffinity Column Washing Solution:

Prepare a mixture of methanol and PBST buffer solution in the ratio of 10:90 (v/v).

5.2. Mobile Phase Preparation

5.2.1. Mobile Phase A:

Prepare a degassed mixture of acetonitrile, methanol and distilled water in the ratio of 1:3:6 (v/v).

5.2.2. Mobile Phase B:

Prepare a degassed mixture of acetonitrile: 0.1% phosphoric acid in ratio 1:1 (v/v)

5.3. Standard Preparation

5.3.1. Intermediate Standard Solution Preparation:

20µL of 50,000ppb (ng/mL) Ochratoxin A standard solution and 400µL of 2600ppb (ng/mL) Aflatoxins standard solution into a 5mL volumetric flask, dilute to volume with methanol and vortex. Concentration of Ochratoxin A is 200ppb (ng/mL) and concentration of Aflatoxins is 208ppb (ng/mL).

5.3.2. Working Standard Solution Preparation:

Prepare working standard solutions using the table below in 5mL volumetric flask with Mobile Phase A.

Working Standard #	Amount of Stock (µL)	Ochratoxin Standard (ppb)	Aflatoxin Standard (ppb)
1	10	0.40	0.42
2	20	0.80	0.83
3	40	1.60	1.66
4	100	4.00	4.16
5	150	6.00	6.24
6	250	10.00	10.40
7	500	20.00	20.80

5.4. Sample Preparation

5.4.1. Sample Extraction

5.4.1.1. Accurately weigh 0.250 g of sample into a 15 ml centrifuge tube.

5.4.1.2. Accurately add 10.0 ml of extraction solution and shake vigorously by hand for 1 minute then vortex for 3 minutes.

5.4.1.3. Centrifuge for 10 minutes at 3500rpm.

5.4.2. Retaining Ochratoxin A and Aflatoxins on Immunoaffinity column

5.4.2.1. Accurately transfer 5 mL of supernatant into sample reservoir.

Note: Pre-load 5 ml of PBST buffer into the sample reservoir.

5.4.2.2. Add PBST buffer to the sample reservoir at the mark of 30mL.

5.4.2.3. Start mycotoxin retaining by gravity or a syringe pump in a flow rate of 1-2 drops/second.

Note: Leave 1-2 mm worth of solution above the top frit of the immunoaffinity column.

5.4.3. Purification of Ochratoxin A and Aflatoxins on Immunoaffinity column

5.4.3.1. Add 30mL of immunoaffinity column washing solution.

5.4.3.2. Clean up the immunoaffinity column with a flow rate of 1-2 drops/second. Drive out all the washing solution and add 10 mL of Milli-Q water.

5.4.3.3. Clean up the immunoaffinity column with a flow rate of 1-2 drops/second. Drive out all the Milli-Q water.

Note: Air should be bubbling through at the end.

5.4.4. Elution of Ochratoxin A and Aflatoxins from Immunoaffinity column

5.4.4.1. Place an HPLC vial under the immunoaffinity column and accurately add 1.5 mL of methanol. Elute mycotoxin by gravity or with a syringe pump.

5.4.4.2. Vortex the elution and inject sample in HPLC system.

5.5. Chromatographic Conditions

Column: Agilent ZORBAX Eclipse XDB-C18, (150 × 4.6) mm, 3.5 µm (Part No. 96397-902)

Flow Rate: 1.2 mL/min.

Detection: Fluorescence Detection for Aflatoxins:
Excitation wavelength: 360 nm
Emission Wavelength: 450 nm
Fluorescence Detection for Ochratoxin A
Excitation wavelength: 330 nm
Emission Wavelength: 460 nm

Column Temperature: 50°C

Injection Volume: 80 µL

Run Time: 27.50 Min.

Pump Mode: Follow Gradient Program

Time (min)	A %	B%
0.00	100.0	0.0
12.00	100.0	0.0
13.00	5.0	95.0
23.00	5.0	95.0
23.10	100.0	0.0
27.50	100.0	0.0

5.6. Sample Set

Inject at least one reagent blank in the beginning of the run. Followed by one injection of each of 7 working standard solutions, be sure to inject working standard # 4 three times. Then inject one reagent blank followed by one injection per sample. End the sample set by injecting one injection of working standard # 4 as Check Standard.

6. Calculations

6.1. $Individual\ Mycotoxin\ (ppb) = \frac{C_{STD}}{A_{STD}} \times \frac{C_{SAM}}{A_{SAM}} \times 1000$

Where: C_{STD} is concentration of standard
C_{SAM} is concentration of sample
A_{STD} is area of standard
A_{SAM} is area of sample

7. References

7.1. Richard JL (2007). "Some major mycotoxins and their mycotoxicoses—an overview". Int. J. Food Microbiol. 119 (1–2): 3–10.

7.2. Bennett, J. W.; Klich, M (2003). "Mycotoxins". Clinical Microbiology Reviews. 16 (3): 497–516.

7.3. National Grain and Feed Association (2011). "FDA Mycotoxin Regulatory Guidance: A Guide for Grain Elevators, Feed Manufacturers, Grain Processors and Exporters." 4.

7.4. Nesheim S, Trucksess MW, Page SW (1999). Molar absorptivities of aflatoxins B1, B2, G1, and G2 in acetonitrile, methanol, and toluene-acetonitrile (9 + 1) (modification of AOAC Official Method 971.22): collaborative study.

7.5. Analytical method development and monitoring of Aflatoxin B1, B2, G1 G2 and Ochratoxin A in animal feed using HPLC with Fluorescence detector and photochemical reaction device, Ho Jin Kim, Mi Jin Lee, Hye Jin Kim, Soon Kil Cho, Hye Jin Park & Min Hee Jeong, Cogent Food & Agriculture (2017), 3: 1419788.

8. Revision History

8.1. HPLC runs were modified to consolidate the testing for the aflatoxins and ochratoxin A into a single run instead of two separate runs. This was to conserve reagents, reduce turnaround time, and preserve the samples; the mycotoxins degrade quickly from light and heat so the more promptly an analysis can be conducted the better.

9. Appendices

N/A