New York State Department of Health - Wadsworth Center
Laboratory of Organic Analytical Chemistry–
NYS ELAP Laboratory ID 10763

Division of Environmental Health Sciences
Albany, New York

Determination of the Plant Growth Regulator Indole-3-butyric Acid and Pesticides in Medical Marijuana using LC-MS/MS

NYS DOH MML-306

Table of Contents

1.0. Scope and Application

1.1. This method (NYS ELAP Method ID 9983) is to be used for the analysis of medical marijuana (MM) products for the determination of residual concentrations of plant growth regulators and selected pesticides (**Table 1**). To be in accordance with Title 10 (Health), Chapter XIII, Part 1004 of the official Compilation of Codes, Rules, and Regulations, of the State of New York, this method will be employed if the use of IBA and or Azadirachtin is divulged by a Registered Organizations (ROs).

Table 1. Analyte List

Analyte	CAS Number	LOD[1] (ng/g) In MCT Matrix	LOQ[2] (ng/g) In MCT Matrix	LOD[1] (ng/ml) In MCT/ Solvent Matrix	LOQ[2] (ng/ml) In MCT/ Solvent Matrix
Indole-3-butyric acid (IBA)	133-32-4	20	100	0.60	3.00
Azadirachtin	11141-17-6	20	100	0.60	3.00
5-Fluoroindole-3-butyric acid (5-FIBA, Internal Standard)	319-72-2	N/A	N/A	N/A	N/A

Note: LODs and LOQs are subject to change.
[1] The Limit of Detection (LOD) is the statistically calculated minimum concentration of an analyte that can be measured with 99% confidence that the value is greater than zero (Section 3.15).
[2] Limit of Quantitation (LOQ) – The minimum concentration that can be quantitatively reported for a target analyte (Section 3.16).

1.2. This method is restricted to use by or under the supervision of analysts experienced in the use of Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Each analyst must demonstrate the ability to generate acceptable results with this method using the procedures described in **Section 11.1**. LOQs referenced within **Table 1** are subject to change based on LOD/LOQ determinations detailed within **Section 11.2.**

2.0. Summary of the Method

2.1. After liquid extraction/dilution with methanol, the samples are analyzed using an LC-MS/MS system operating in the negative ion electrospray ionization (ESI) mode with multiple reaction monitoring (MRM) for specific detection of the analyte and IS. Chromatography of IBA, Azadirachtin, and 5-FIBA is performed on a C_{18} reverse-phase column using a programed gradient of increasing organic modifier over a period of 15 minutes. See Table 1 for limits of detection (LOD) and limit of quantification (LOQ) for IBA and Azadirachtin.

3.0. Definitions

3.1. Internal Standard (**IS**) – A pure compound that is not found in any sample. The IS is a compound added to unknown samples, Quality Control (QC) samples, including method blanks, laboratory fortified blanks, matrix spikes, duplicates, and calibration standards at a known concentration in order to provide a basis for peak area ratios used in quantitation. The IS is also used to monitor instrument performance for each analysis.

3.2. Internal Standard Stock Diluent (**ISD**) – A concentrated solution of IS that is prepared in extraction solvent. This stock diluent is used to prepare the IS working diluent (IWD).

3.3. Internal Standard Working Diluent (**IWD**) – A solution of IS that is prepared in extraction solvent from the ISD that is added to all samples at the same concentration. This working diluent is used to dilute the samples and to monitor the integrity of the sample injections.

3.4. System Blank **(SBLK)** – A portion of an appropriate pure solvent that is analyzed to verify that the instrument is free from background contamination.

3.5. Laboratory Reagent Blank (**LRB)** – An aliquot of extraction solvent that is treated exactly as if it were a sample, including exposure to all glassware, equipment, solvents, internal standards, and reagents that are used with the samples. The **LRB** is used to determine whether method analytes or other interferences are present in the laboratory environment, reagents or apparatus. Synonyms: Method Blank

3.6. Laboratory Fortified Blank (**LFB**) – An aliquot of sample matrix, free from the analytes of interest, spiked with verified known amounts of analytes or a material containing known and verified amounts of analytes and taken through all sample preparation and analytical steps of the procedure unless otherwise noted in a reference method. It is generally used to establish intra-laboratory or analyst specific precision and bias or to assess the performance of all or a portion of the measurement system. Synonyms: Method Blank Spike (MBS) and Laboratory Control Sample (LCS).

3.7. Matrix Blank (**MB**) – An aliquot of matrix that is treated exactly as if it were a sample, including exposure to all glassware, equipment, solvents, internal standards, and reagents that are used with the samples. The **MB** is analyzed to verify that there are no interfering peaks arising from the matrix.

3.8. Matrix Spike (**MS**) – An aliquot of sample prepared, taken through all sample preparation and analytical steps of the procedure unless otherwise noted in a referenced method, by adding a known amount of target analyte to a specified amount of sample for which an independent test result of target analyte concentration is available. Matrix spikes are used, for example, to determine the effect of the matrix on a method's recovery efficiency. When sample is not available, a mock matrix may be used instead. Synonym: Laboratory Fortified Sample Matrix (LFM).

3.9. Matrix Spike Duplicate (**MSD**) – Prepared identically to the **MS**, the MSD is a second portion of actual sample. It is spiked and processed in an identical manner to that of the **MS**. The **MS** and **MSD** are used together to evaluate the precision of the methodology.

3.10. Primary Stock Standard (**PS**) – A concentrated solution of method analyte(s) prepared in the laboratory from referenced and certified analyte standards, or a concentrated solution of method analyte(s) purchased directly from a referenced and certified source.

3.11. Primary Working Standard (**WS**) – A solution of the method analyte(s) prepared from stock standard solutions that is diluted as necessary to prepare calibration standards or other necessary analyte solutions. Synonym: Primary Dilution Standard Solution (PDS).

3.12. Calibration Standard (**CalS**) – A solution of method analytes prepared from stock or working standard solutions that is used to calibrate the instrument response with respect to analyte concentration.

3.13. Continuing Calibration Verification Standard (**CCV**) – One of the calibration standards used to verify the acceptability of an existing calibration. Synonyms: Continuing Calibration Check Standard (CCC).

3.14. Cross Check Reference Standard (**CCR**) – A solution of method standards prepared from a stock standard solution that is obtained from a source that is independent of that used to prepare the calibration standards (i.e., independent vendor, independent lot, or independent preparation). The CCR is used to verify that the original calibration source is acceptable.

3.15. Limit of Detection **(LOD)** – The statistically calculated minimum concentration of an analyte that can be measured with 99% confidence that the value is greater than zero. Synonym: Method Detection Limit (MDL).

3.16. Limit of Quantitation (**LOQ)** – The minimum concentration that can be quantitatively reported for a target analyte. For routine analyses, the lowest calibration standard must be at or below the **LOQ** for each analyte. **LOQ** is typically 3-5 times the **LOD**. Synonym: Method Reporting Limit (MRL).

3.17. Upper Limit of Quantitation (**ULOQ**) – The maximum acceptable point on the calibration curve. The **ULOQ** is the concentration of the most concentrated **CalS** standard.

3.18. Preparation Batch - Samples that are prepared and/or analyzed together with the same process and personnel, using the same lot(s) of reagents. A preparation batch consists of one to twenty samples (not including method blanks, LCS, matrix spikes and matrix duplicates) of the same matrix with a maximum processing time of twenty-four (24) hours between the first and last sample.

3.19. Analytical Batch **–** An analytical batch consists of prepared samples which are analyzed together as a group. An analytical batch can include prepared samples originating from different matrices and can exceed twenty samples.

4.0. Health and Safety Warnings

4.1. Caution must be used when working with IBA, Azadirachtin, and 5-FIBA. According to the Safety Data Sheets (SDSs), IBA is hazardous for skin contact (irritant), eye contact (irritant), ingestion and inhalation. Those working with IBA, Azadirachtin and 5-FIBA must always wear gloves, labcoats and safety glasses, and operate in a well-ventilated hood when working with these materials. Immediate handwashing following the handling of standards and samples is greatly encouraged.

4.1. The toxicity and carcinogenicity of each chemical used in this method have not been thoroughly investigated. Therefore, each chemical compound used in this method must be treated as a potential health hazard, and exposures must be limited to the lowest possible levels.

4.2. Always follow guidelines listed in SDSs for proper storage, handling, and disposal of solvents, reagents, and standards. SDSs are located within the laboratory in labeled, yellow binders. These guidelines must be made available to all personnel involved in the chemical analyses.

4.3. Lab coats, safety glasses and gloves must be worn when performing all standard or sample preparations and when working with instrumentation, disposing of waste, and cleaning glassware.

4.4. The fume hood must be used when using or preparing standards, reagents, or samples that require proper ventilation.

5.0. Interferences

5.1. Method interferences may be caused by contaminants in solvents and reagents, on glassware, and other equipment used in sample processing, and may be manifested as discrete peaks or elevated baselines in the chromatograms. All reagents and apparatus must be routinely demonstrated to be free from interferences under the conditions of the analysis by running a **LRB** as described in **Section 11.4.**

5.1.1. Glassware and syringes used in the medical marijuana lab must be thoroughly cleaned in order to prevent contamination. After use, rinse with the last solvent used, then rinse three times with dichloromethane, three times with acetone, and three times with methanol.

5.1.2. The use of high-purity reagents and solvents helps to minimize problems with interferences. Purification of solvents by distillation is not performed in this laboratory, nor is it required.

5.1.3. After cleaning, glassware is stored in a clean cabinet away from standards and syringes to prevent and cross-contamination.

5.2. When interferences or contamination are evident in a sample, the re-preparation of the original sample is recommended after the source of contamination is identified and eliminated.

5.3. Interfering contamination due to "carry over" may occur when a sample containing low concentrations of analytes is analyzed immediately following a sample containing relatively high concentrations of analytes. Rinsing of the autosampler syringe and associated equipment with needle wash (methanol) can minimize sample cross contamination.

5.3.1. If target analytes are present at an unusually high concentration of extracted sample, the analyst must demonstrate that the analytes in the subsequent samples are not due to carry over. In addition, after analysis of a sample containing high concentrations of analytes, one or more injections of Lab Reagent Blank (**LRB; section 12.3**) should be made to ensure that there is no carry over, and that accurate values are obtained for the next sample. The LRB must pass contamination criteria set in **section 11.4.2**.

5.3.2. Alternately, if the samples immediately following the high concentration sample do not contain the analytes that were at high concentration (calculated concentration < LOD), freedom from contamination has been established.

5.4. Matrix interferences may occur as a result of contaminants present in the sample. If matrix interference is believed to have occurred, it is recommended that a matrix spike be analyzed with the sample to verify results. This may not always be possible given the amount of sample that is received for analysis.

5.5. Samples, QC samples, and standards must be prepared in the same final solvent to allow for chromatographic comparability of samples and standards.

6.0. Instrumentation, Equipment and Supplies

(All specifications are suggested. Catalog numbers are included for illustration only.)

6.1. Standard and Sample Preparation Equipment

6.1.1. Class "A" volumetric flasks with stoppers, various sizes.

6.1.2. Positive pipette and different size of pipette tips.

6.1.3. 20 mL glass scintillation vials, or equivalent.

6.1.4. 1.5-ml auto-sampler vials with 0.3-ml target poly-spring inserts and Teflon-lined screw-caps or crimp-top caps.

6.1.5. Micro pipette controller, various sizes, Eppendorf, Research Plus, or equivalent.

6.2. Sample Extraction Equipment

6.2.1. Analytical balance, Mettler-Toledo, model # XSE205DU, or equivalent.

6.2.2. 2-mL centrifuge tubes.

6.2.3. Vortex – ThermoLyne, Maxi Mix 11, model #37615, or equivalent.

6.2.4. Shaker, Labline, Model # 3540 or equivalent.

6.2.5. Centrifuge – Eppendorf, model # 5415D, or equivalent.

6.2.6. Sonicator – Branson, model # 2510R-DTH, or equivalent.

6.3. Instrumentation

6.3.1. An LC-MS/MS system with all of the required accessories including: syringes, analytical columns, mobile phases, detectors, and a data system (e.g., a Shimadzu HPLC interfaced with an AB Sciex triple quadrupole mass spectrometer, or equivalent). The mass spectrometer system must be capable of running multiple reaction monitoring (MRM) instrumental methods, or an equivalent type of method.

6.3.1.1. Shimadzu HPLC system includes:

6.3.1.1.1. Micro vacuum degasser; model # DGU-20A5 or equivalent

6.3.1.1.2. Pumps; model # LC-20ADxR, or equivalent

6.3.1.1.3. Column oven; model # CTO-20A or equivalent

6.3.1.1.4. Autosampler; model # SIL-20ACxR or equivalent

6.3.1.1.5. Solvent selector; model# FCV-11AL or equivalent

6.3.1.1.6. System controller; model # CBM-20A or equivalent

6.3.1.1.7. HPLC column; Agilent Poroshell 120 EC-C18, 2.7 µm particle size, 3.0 x 150 mm column, #693975-302, or equivalent.

6.3.1.2. Triple quadrupole or QTRAP mass spectrometer system, which includes:

6.3.1.2.1. AB Sciex 4500 Mass Spectrometer (MS/MS); model # API-4500 QTRAP, or equivalent.

6.3.1.2.2. The AB Sciex 4500 QTRAP is equipped with a Turbo Ion Spray Ion Source

6.3.1.2.3. Analyst Software; version 1.6.1, or equivalent.

6.3.1.2.4. Nitrogen gas for use as the nebulizing gas, turbo gas, and collision cell gas; Airgas, Cryogenic Liquid Nitrogen, NI 265LT350, or equivalent.

7.0. Reagents and Standards (Consumables)

7.1. Solvents and Reagents

7.1.1. Methanol – (HPLC grade, J.T. Baker, catalog # 9830-03 or equivalent).

7.1.2. HPLC water – (HPLC grade, Sigma Aldrich, catalog # 34877-4L or equivalent).

7.1.3. Ammonium formate – (High purity (98 % +), Fluka catalog # 3272-02 or equivalent).

7.1.4. Formic acid – (MSD grade, Sigma-Aldrich catalog # 39,938-8 or equivalent).

7.1.5. Dichloromethane – (Sigma-Aldrich, Catalog # 650463-4L, or equivalent)

7.1.6. Acetone – (Mallinckrodt, Catalog # 2432, or equivalent).

7.1.7. Medium Chain Triglycerides (MCT Oil) – (Warner Graham, Miglyol 812, or equivalent).

7.2. Stock Analytical and Internal Standard Solutions

7.2.1. Stock standards may be purchased from any vendor. Standards are preferably to be ISO Guide 31 and 35 accredited and NIST traceable, when available.

7.2.2. The commercial standards listed below (Table 2) are examples of those currently used in the laboratory. At a minimum, commercial standards are stored per the manufacturer's recommended storage conditions, and expiration dates of commercially prepared standards are as prescribed by the vendor on their Certificate of Analysis.

Table 2. Analytical Standards

Standard	Manufacturer	Catalog #	Concentration	Solvent
Indole-3-butyric acid (IBA)	Sigma-Aldrich	45532-250MG	N/A	Solid
Azadirachtin (AZ)	Sigma-Aldrich	A7430-17MG	N/A	Solid
Azadirachtin (AZ)	LGC	DRE-GA09011042AL	100 µg/mL	ACN
5-Fluoroindole-3-butyric acid (5-FIBA, IS)	Oakwood Chemical	040689-1G	N/A	Solid

8.0. Preparation of Reagents, Solutions, and Standards

8.1. General Preparation Information

8.1.1. All reagents, solutions and standards must be traceable to stocks, and if possible, have NIST-traceable documentation. The preparation method, date of preparation, expiration date, and analyst must be traceable in laboratory documentation.

8.1.2. Standards labeled below are for guidance only. These may be interchanged. In addition, different concentrations or analyte stock mixtures may be prepared as necessary.

8.1.3. To ensure that an accurate amount of diluent is transferred, mix each solution in the auto-sampler vials by drawing up and dispensing diluent at least 5 times before transferring. Diluent may be dispensed back into the container from which it was drawn.

8.2. Extraction Solvent

8.2.1. Pure HPLC grade methanol.

8.2.2. Solution is stable for 12 months at room temperature after opening.

8.3. Internal Standard (**IS**) – @ 1.00 mg/mL for 5-FIBA.

8.3.1. Accurately weigh 10.0 mg (± 0.1 mg) reference standard of 5-FIBA in a 20 mL scintillation vial.

8.3.2. Add 10.0 mL HPLC grade methanol using a positive pipette, cover, and vortex to dissolve the solid **IS** completely.

8.3.3. Aliquot of IS solution is transferred and sealed in a 1.5-mL crimp-cap amber vial and labeled.

8.3.4. Store at -80 °C for up to 12 months, in a sealed vial or ampule.

8.4. Internal Standard Working Diluent **(IWD)**

8.4.1. Add 40.0 µL of **IS** @ 1.00 mg/mL (**Section 8.3**).

8.4.2. Dilute with 9.96 mL HPLC grade methanol and vortex to mix.

$$(1000\ \mu g/mL)\ (0.040\ mL) = (X\ \mu g/mL)\ (10.0\ mL)$$

$$X = 4.00\ \mu g/mL$$

8.4.3. Solution is transferred and sealed in 1.5 mL crimp-cap vial and labeled.

8.4.4. Store at -80 °C for up to 12 months, in a sealed vial or ampule.

8.5. Primary Stock Standards (**PS**) – @ 1.00 mg/mL for IBA.

8.5.1. Accurately weigh 10.0 mg (± 0.1 mg) reference standard of IBA in a 20 mL scintillation vial.

8.5.2. Add 10.0 mL HPLC grade methanol using a positive pipette, cover, and vortex to dissolve the solid completely.

8.5.3. An aliquot of **PS** solution is transferred and sealed in a 1.5-mL crimp-cap amber vial and labeled.

8.5.4. Store at -80 °C for up to 12 months, in a sealed vial or ampule.

8.6. Secondary Stock Standard – @ 1.00 mg/mL for IBA.

8.6.1. Accurately weigh another 10.0 mg (± 0.1 mg) reference standard of IBA in a 20 mL scintillation vial.

8.6.2. Add 10.0 mL HPLC grade methanol using a positive pipette, cover, and vortex to dissolve the solid completely.

8.6.3. An aliquot of this solution is transferred and sealed in a 1.5-mL crimp-cap amber vial and labeled.

8.6.4. Store at -80 °C for up to 12 months, in a sealed vial or ampule.

8.7. Primary Stock Standards (**PS**) – @ 1.00 mg/mL for Azadirachtin.

8.7.1. Accurately weigh 10.0 mg (± 0.1 mg) reference standard of Azadirachtin in a 20 mL scintillation vial.

8.7.2. Add 10.0 mL HPLC grade methanol using a positive pipette, cover, and vortex to dissolve the solid completely.

8.7.3. An aliquot of the **PS** solution is transferred and sealed in a 1.5-mL crimp-cap amber vial and labeled.

8.7.4. Store at -80 °C for up to 12 months, in a sealed vial or ampule.

8.8. Secondary Stock Standard – @ 1.00 mg/mL for Azadirachtin.

8.8.1. Accurately weigh 5.00 mg (± 0.05 mg) reference standard of Azadirachtin in a 20 mL scintillation vial.

8.8.2. Add 5.00 mL HPLC grade methanol using a positive pipette, cover, and vortex to dissolve the solid completely.

8.8.3. An aliquot of the solution is transferred and sealed in a 1.5-mL crimp-cap amber vial and labeled.

8.8.4. Store at -80 °C for up to 12 months, in a sealed vial or ampule.

8.9. Primary Working Standard (**WS**) – @ 1000 ng/mL for IBA and Azadirachtin.

8.9.1. Add 10.0 µL of IBA **PS** @ 1.00 mg/mL (**Section 8.5**) and 10.0 µL of Azadirachtin **PS** @ 1.00 mg/mL (**Section 8.7**).

8.9.2. Dilute with 9.98 mL HPLC grade methanol and vortex to mix.

$$(1000\ \mu g/mL)\ (0.010\ mL) = (X\ \mu g/mL)\ (10.0\ mL)$$

$$X = 1.00\ \mu g/mL$$

8.9.3. This solution is transferred and sealed in a 1.5-mL crimp-cap amber vial and labeled.

8.9.4. Store at -80 °C for up to 12 months, in a sealed vial or ampule.

8.10. Secondary Working Standard – @ 1000 ng/mL for IBA and Azadirachtin.

8.10.1. Add 10.0 µL of IBA **Secondary Stock Standard** @ 1.00 mg/mL (**Section 8.6**) and 10.0 µL of Azadirachtin **Secondary Stock Standard** @ 1.00 mg/mL (**Section 8.8**).

8.10.2. Dilute with 9.98 mL HPLC grade methanol and vortex to mix.

$$(1000\ \mu g/mL)\ (0.010\ mL) = (X\ \mu g/mL)\ (10.0\ mL)$$

$$X = 1.00\ \mu g/mL$$

8.10.3. This solution is transferred and sealed in a 1.5-mL crimp-cap amber vial and labeled.

8.10.4. Store at -80 °C for up to 12 months, in a sealed vial or ampule.

8.11. Cross check reference standard working solution (**CCR-WS**) @ 250 ng/ml.

8.11.1. Add 250 µL of Secondary Working Standard @ 1000 ng/ml (**Section 8.10**).

8.11.2. Dilute with 750 µL HPLC grade methanol and vortex to mix.

8.11.3. This solution is transferred and sealed in a 1.5 mL crimp-cap amber vial and labeled.

8.11.4. Store at -80 °C for up to 12 months, in a sealed vial or ampule.

8.12. Mobile Phases

8.12.1. Mobile phase A: 5mM ammonium formate, 0.02 % formic acid in HPLC water.

8.12.1.1. 0.315 g of ammonium formate is added to a scintillation vial and diluted with a small volume of HPLC-grade water.

8.12.1.2. Ammonium formate solution prepared in section 8.12.1.1 is transferred to a 1-L volumetric flask. The scintillation vial is rinsed well with HPLC-grade water, and 0.200 mL of formic acid is added to the 1-L volumetric flask. Dilute to volume with HPLC-grade water and mix well.

8.12.1.3. This solution must be prepared monthly.

8.12.2. Mobile phase B: Methanol.

8.12.2.1. This solution must be changed at least every 6 months.

8.13. Matrices

8.13.1. MCT as a representative matrix.

8.13.1.1. MCT matrix will be used for the Demonstration of Capability (DOC) and matrix spike.

9.0. Sample Transport, Receipt, Preservation, and Storage

9.1. Sample transport conditions:

9.1.1. The MM products from the ROs are shipped as per manufacturer's specifications and must adhere to all regulatory requirements.

9.2. Sample receipt:

9.2.1. Medical marijuana products from the RO are received, verified and documented ensuring that method, regulatory and Accreditation Body requirements are met.

9.2.2. All MM products must be stored under the conditions recommended by the manufacturer(s). The storage is documented.

9.3. Preservation:

9.3.1. All samples prepared for analysis are placed in sealed containers and refrigerated at ≤ 4 °C for storage, for no longer than one week, and they must be kept away from light until analysis.

9.4. Extract Storage:

9.4.1. Sample extracts are stored in a freezer at ≤ -20 °C until analysis, which must be completed within 7 days of extraction. The samples are warmed to room temperature and vortexed to ensure homogeneity before analysis. After analysis is complete, the remaining extract is stored at ≤ -20 °C for one month if necessary.

10.0. Calibration

10.1. LC-MS/MS

10.1.1. The m/z scale and resolution of the ESI mass spectrometer must be periodically calibrated with the tuning solution and procedures prescribed by the manufacturer.

10.1.2. Optimize the MS/MS parameters, including collision energies (CE), declustering potential, gas flows, and temperatures according to the manufacturer's instructions. An example of optimal MS/MS conditions are described in **Tables 3** and **4**.

Table 3: Instrument specific MS/MS parameters:

Parameter	Value
MS Acquisition Time:	7.0 – 15.0 min.
Curtain Gas Flow (CUR):	30.00 psi
Collision Gas Pressure (CAD Gas):	Medium
Ion Transfer Voltage (IS):	-4500.00 V
Temperature of Turbo Gas (TEM):	550.00 ºC
Gas 1 – Nebulizer Gas (GS1):	40.00 psi
Gas 2 – Turbo Gas (GS2):	60.00 psi
Declustering Potential Voltage (DP):	-45.00 V
Entrance Potential Voltage (EP):	-10.00 V
Collision Cell Exit Potential (CXP):	-13.00 V

Table 4: MS/MS Analyte-specific parameters.
Quantifying product ions and CE are **bold**, qualifying product ions and CE are in (parenthesis).

Analytes	Polarity	Precursor ion *m/z*	Product ion *m/z*	Collision energy (eV)	Dwell time (msec)
IBA	Negative	202	**116** (158)	**-25** (-17)	50
Azadirachtin	Negative	719	**659** (687)	**(-20)** (-15)	50
5-FIBA, (IS)	Negative	220	**134** (147)	**-50** (-18)	50

10.1.3. Please note that the parameters above have been optimized to give the best signal-to-noise ratios for the determination of analyte and IS present in matrix extracts, not necessarily to give the best signal-to-noise ratios for their determination in solvent.

10.1.4. Using the MS/MS parameters above and the LC operating parameters described in **Table 5**, analyze a mid-level calibration standard to obtain retention times (**Table 6**) for the target analyte using an MRM instrumental method, or equivalent. **Figure 1** is an example of a chromatogram. For optimal MS/MS precision, there must be at least 10 scans across each peak.

Table 5: HPLC parameters

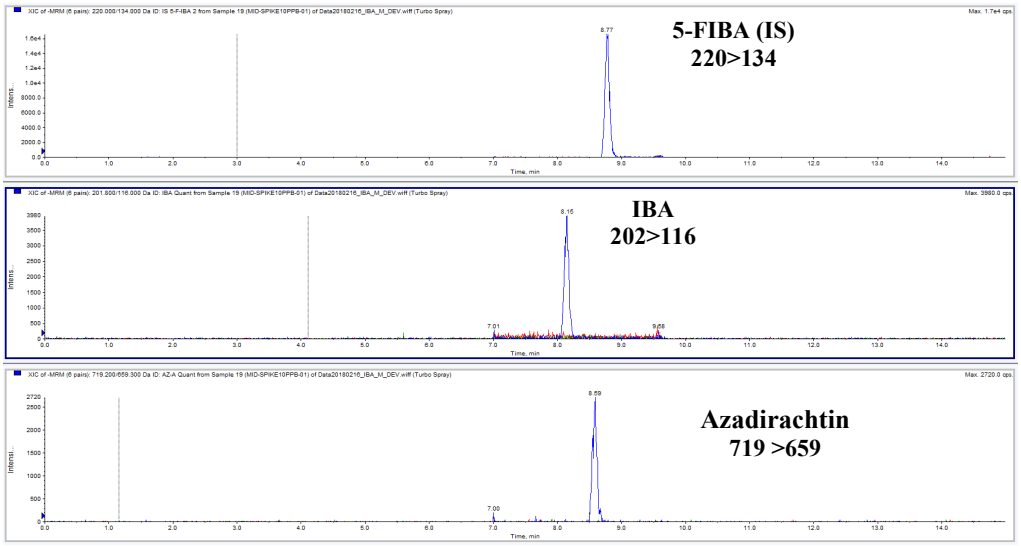
Column	**Value**
Column:	Agilent Poroshell 120 EC-C18, 2.7 µm, 3.0 x 150 mm, # 693975-302
Guard Column:	Agilent Poroshell 120 EC-C18, 2.7 µm, 3.0 x 5 mm, #823750-911
Column Temperature:	40.0 °C
Autosampler	**Value**
Temperature:	4.0 °C
Injection Volume:	10.0 µL
Injection Loop Volume:	50.0 µL
Rinse Mode:	Before and after aspiration
Rinse Volume:	200 µL
Rinse Solvent:	Methanol
Mobile Phases	**Value**
Mobile Phase A:	5mM ammonium formate, 0.02 % formic acid in H_2O
Mobile Phase B:	Methanol
Flow Rate:	0.400 mL/min
Initial Percentage MPB:	10%
Diverter Valve - Time	**Flow Destination (Position)**
Initial:	Divert to waste (Position A)
7.0 min:	Divert to MS detector (Position B)
9.5 min:	Divert to waste (Position A)
HPLC Gradient, Time	**Percentage of Mobile Phase B**

Initial:	10%
1.0 min:	10%
2.0 min:	50%
10.0 min:	70%
11.0 min:	95%
15.0 min:	95%
15.1 min:	10%
20.0 min:	10% (End of Run)

Table 6: Representative Analyte and IS Retention Times

Analyte or IS	Expected Retention Time (min)	Retention Time Range (min)
IBA	8.15	8.05 – 8.25
Azadirachtin	8.60	8.50 – 8.70
5-FIBA (IS)	8.80	8.70 – 8.90

Figure 1. Chromatogram of the medium level of spiked extraction (10.0 ng/mL IBA and Azadirachtin spike) in MCT matrix. The 10.0 ng/mL in MCT matrix (blue), is shown overlaid on the chromatogram recorded for the solvent blank (red), and the MCT matrix blank (green).



10.2. Calibration Curve Preparation (**CalS**)

10.2.1. Serial dilutions are made from the Primary working solution of IBA and Azadirachtin (**Section 8.9**). (The concentration range is 3.90 ng/mL to 1000 ng/mL.) (see Table 7) Suggested storage for standards is ≤ -80 °C for up to 12 months, in a sealed vial or ampule.

10.2.1.1. ***CalS 5a*** (1000 ng/mL)

10.2.1.2. CalS 5a is the same as the Primary Working Standard (**Section 8.9**).

10.2.2. ***CalS 4a*** (250 ng/mL)

10.2.2.1. Dispense 250 µL of **CalS5a** (**Section 10.2.1**) into a vial, labeled **CalS 4a,** containing 750 µL of methanol.

10.2.2.2. Vortex to mix well.

(1000 ng/mL) (250 µL) = (X ng/mL) (1000 µL)

X= 250 ng/mL

10.2.3. ***CalS 3a*** (62.5 ng/mL)

10.2.3.1. Dispense 250 µL of **CalS 4**a (**Section 10.2.2**) into a vial, labeled **CalS 3a,** containing 750 µL of methanol.

10.2.3.2. Vortex to mix well.

(250 ng/mL) (250 µL) = (X ng/mL) (1000 µL)

X= 62.5 ng/mL

10.2.4. ***CalS 2a*** (15.6 ng/mL)

10.2.4.1. Dispense 250 µL of **CalS 3a** (**Section 10.2.3**) into a vial, labeled **CalS 2a,** containing 750 µL of methanol.

10.2.4.2. Vortex to mix well.

(62.5 ng/mL) (250 µL) = (X ng/mL) (1000 µL)

X= 15.6 ng/mL

10.2.5. ***CalS 1a*** (3.90 ng/mL)

10.2.5.1. Dispense 250 µL of **CalS 2a** (**Section 10.2.4**) into a vial, labeled **CalS 1a,** containing 750 µL of methanol.

10.2.5.2. Vortex to mix well.

(15.6 ng/mL) (250 µL) = (X ng/mL) (1000 µL)

X= 3.90 ng/mL

Table 7. – Calibration Curve without IS

CalS STD	Std Conc. (ng/mL)	Std Ref Section ID	Volume of STD	Extraction Solvent (Section 8.2)
CalS 5a	1000	10.2.1	-	-
CalS 4a	250	10.2.2	250 µL	750 µL
CalS 3a	62.5	10.2.3	250 µL	750 µL
CalS 2a	15.6	10.2.4	250 µL	750 µL
CalS 1a	3.90	10.2.5	250 µL	750 µL

10.2.6. Prepare calibration standard mixtures with **IWD** by diluting 100 µL of each **CalS** (**Section 10.2.1 – 10.2.5**) with 10.0 µL of **IWD** @ 4.00 µg/mL (**Section 8.4**) and 900 µL methanol (**Section 8.2**) mixing well. Be sure to rinse solution down the sides of the container. Failure to mix well will cause a failure of linearity requirements. The final concentrations are provided in **Table 8**.

Table 8. – Final Calibration Curve with IS (for analysis)

Cal STD w/IWD	Final Concentration (ng/mL)	Std Ref Section ID	Volume of IBA STD without IS	IWD Ref ID	Volume of IWD	Methanol (Section 8.2)	IS Final Concentration (ng/mL)
CalS 5b	100	10.2.1	100 µL	8.4	10 µL	900 µL	40.0
CalS 4b	25	10.2.2	100 µL	8.4	10 µL	900 µL	40.0
CalS 3b	6.25	10.2.3	100 µL	8.4	10 µL	900 µL	40.0
CalS 2b	1.56	10.2.4	100 µL	8.4	10 µL	900 µL	40.0
CalS 1b	0.390	10.2.5	100 µL	8.4	10 µL	900 µL	40.0

10.2.7. After at least one system blank injection, start with the lowest standard concentration (**CalS 1b**) and analyze each calibration standard. Tabulate the response (peak area/IS peak area ratio) and use the result to prepare a calibration curve for each target analyte (weighted 1/X linear regression).

10.3. Initial Calibration Criteria

10.3.1. The solutions prepared in **Section 10.2** are used to prepare a calibration curve for IBA and Azadirachtin at concentrations appropriate for the instrument's range and sample content. A minimum of 5 calibration concentrations is used for each analyte.

10.3.2. The lowest calibration standard must be at or below the **LLOQ** listed in **Section 1.1** for the analyte, or the **LLOQ** must be adjusted accordingly.

10.3.3. The correlation coefficient (R) of the calibration curve for the analyte must be ≥ 0.995 before any analysis of samples can begin.

10.3.4. Each calibration standard, processed under the new initial calibration, must be within 80-120% of the true value for each analyte for the initial calibration to be considered valid. The exception is the lowest calibration point, which may be within 70-130% of the true value for each analyte. Evaluation of each standard also serves as the measure of % Relative Error with the same acceptance criteria.

10.3.5. If all criteria cannot be met a new calibration must be established.

10.4. Initial Verification of Calibration

10.4.1. The initial calibration for IBA and Azadirachtin must be verified by analyzing a Cross Check Reference Standard (**CCR**) at a mid-level concentration. To prepare **CCR** @ 25.0 ng/mL:

10.4.1.1. Dispense 100 µL of **CCR-WS** @ 250 ng/mL (**Section 8.11**) into a vial.

10.4.1.2. Add 10.0 µL of **IWD** @ 4.00 µg/mL (**Section 8.4**) and 900 µL methanol (**Section 8.2**)

10.4.1.3. Mix well. The final concentration is now 25.0 ng/mL analytes, and 40.0 ng/mL **IS**.

10.4.2. Measured recovery value of **CCR** must be within 80 – 120% of the true value for IBA and Azadirachtin.

10.5. Continuing Calibration Check

10.5.1. After verifying the initial calibration, a CCV that is ≤ 1/2 the highest calibration standard must be analyzed with each analytical batch (typically 20 samples). For external calibration, a CCV is required at the beginning and end of each analytical batch. For internal standard calibration, a CCV is only required at the beginning of the analytical batch.

10.5.1.1. Low-level CCVs that are ≤ the LOQ must be within 70-130 % of the known value for each analyte. CCVs > the LOQ must be within 80-120% of the predicted concentration.

10.5.2. CCVs may also be interspersed throughout the analytical batch at varying concentrations provided that the CCVs analyzed at the beginning and end (for external calibration) of each analytical batch are equal to or less than half the highest calibration level. Additional CCVs may also be run at higher levels to evaluate the upper end of the calibration curve.

10.5.3. Examples of CCVs are prepared at the following levels:

10.5.3.1. 1.56 ng/mL, 6.25 ng/mL, and 25 ng/mL (same as **CalS 2b** thru **4b** – (**Section 10.2 Table 8**). LOQ standard for IBA and Azadirachtin is at 0.390 ng/mL in extraction solvent (same as **CalS 1b**).

11.0. Quality Control/Assurance

11.1. Demonstration of Capability (DOC)

11.1.1. Each analyst must perform an initial demonstration of capability using the procedures described in this SOP for the target analytes. The initial DOC must consist of the analysis of four or five matrix spike samples that have been fortified with target analytes at a concentration of one (1) to four (4) times the LOQ. The spiking solution used must be from a source that is independent of the standards used to prepare the calibration curve, if one is available.

11.1.1.1. To determine the accuracy of the analytes, the individual recovery of each replicate must be within ± 20% of the true value. The precision of the measurements, calculated as relative standard deviation (RSD), must be 20% or less. When analyses fail to meet these criteria, this procedure must be repeated until satisfactory performance has been demonstrated.

11.1.1.2. Annually, each analyst must complete a continuing DOC. To determine the accuracy of the analytes, the individual recovery of each replicate must be within ± 20% of the true value. The precision of the measurements, calculated as relative standard deviation (RSD), must be 20% or less. When analyses fail to meet these criteria, this procedure must be repeated until satisfactory performance has been demonstrated. The continuing DOC may be completed by one of the following techniques:

11.1.1.2.1. Acceptable performance of a blind sample.

11.1.1.2.2. Acceptable performance by interlaboratory comparison of samples, where available.

11.1.1.2.3. Acceptable performance of an initial DOC as described above in 11.1.1 at any concentration within the calibration range.

11.1.2. If major changes to the method or instrument are made, or the laboratory/analyst has not performed the method in a twelve (12) month period, each analyst must complete an initial DOC as described in **Section 11.1.1**. Minor changes to the method are evaluated using the matrix spike per **Section 11.7** for routine samples or the secondary source standard per **Section 10.4**.

11.2. Limit of Detection (LOD)

11.2.1. An initial LOD study for each method must be completed and documented for all target analytes in each representative matrix (see **MML-301-SOP, Section 7.3**), on each

instrument used to analyze sample extracts. If the laboratory intends to report results below the LOQ, an ongoing LOD verification is also required.

11.2.2. Based on the LOD, the laboratory shall select an LOQ that is greater than the LOD (typically 3-5x the LOD) and consistent with the needs of its client. An LOQ is required for each representative matrix, method and analyte combination. For each method, the lowest calibration standard concentration must be at or below the corresponding LOQ.

11.2.3. An initial LOQ study for each method must be completed and documented for all target analytes in each representative matrix. The initial LOD samples may be used for this purpose as long as the concentration used is at or below the LOQ. The mean recovery shall be within 70-130% of the spiked value.

11.2.4. On an ongoing basis, the laboratory shall prepare and analyze a minimum of one LOQ verification sample spiked at the same concentration as the initial LOQ verification study on each instrument during each quarter in which samples are being analyzed for each representative matrix, method, and analyte combination. The recovery of the LOQ verification samples shall be within 70-130%.

11.2.5. The 2017 Method Update Rule finalized in the Environmental Protection Agency's (EPA's) Federal Register on August 28, 2017, prescribes a revised approach to Method Detection Limit (MDL)/LOD data collection and calculation per Part 136 Appendix B. The New York State (NYS) Environmental Laboratory Program (ELAP) requires that the revised procedure detailed within the EPA's document *Definition and Procedure for the Determination of the Method Detection Limit, Revision 2, December 2016* be implemented for all NYS ELAP accredited methods.

11.3. System Blank (**SBLK**)

11.3.1. Before processing samples, the analyst must demonstrate that the instrument is free from background interference by analyzing a system blank (**SBLK**).

11.3.1.1. For our analysis, a vial of extraction solvent (**Section 8.2**) will be injected.

11.3.2. Background contamination, if it is observed and is such that it could interfere with the measurement of target analyte(s), must be < 1/3 **LOQ** for routine samples.

11.4. Laboratory Reagent Blank (**LRB**)

11.4.1. Before processing samples, the analyst must demonstrate that all interferences arising from glassware and reagents are under control. Each time a set of samples is extracted or reagents are changed, or with every twenty samples, whichever is more frequent, a **LRB** must be analyzed. If, within the relative retention time window of the target analyte, the **LRB** produces a peak that would prevent the determination of the analyte, the source of the interference must be determined and eliminated before processing the samples.

11.4.2. Background contamination found must be <1/3 of the **LOQ** for the target analyte.

11.5. Laboratory Fortified Blank (**LFB**)

11.5.1. The laboratory must analyze at least one laboratory fortified blank (**LFB**) with every preparation batch (one to twenty samples of the same matrix with a maximum processing time of twenty-four (24) hours between the first and last sample).

11.5.2. . The **LFB** is fortified with the target analyte at a mid-level concentration.

11.5.3. The accuracy is calculated as percent recovery. The recovery for the analyte must be 80 – 120% of the true value.

11.6. Matrix Blank (**MB**)

11.6.1. The laboratory must analyze at least one matrix blank (**MB**) with every preparation batch (one to twenty samples of the same matrix with a maximum processing time of twenty-four (24) hours between the first and last sample).

11.6.2. If, within the relative retention time window of any target analyte, the **MB** produces a peak that would interfere with the determination of the analyte, refer to laboratory specific procedures.

11.6.3. Background contamination found must be <1/3 the **LOQ** for the target analyte.

11.7. Matrix Spike and Matrix Spike Duplicate (**MS and MSD**)

11.7.1. A matrix spike sample must be analyzed with each preparation batch (one to twenty samples of the same matrix with a maximum processing time of twenty-four (24) hours between the first and last sample).

11.7.2. The **MS** is fortified with the target analyte at a mid-level (10 ng/mL) concentration.

11.7.3. A duplicate matrix spike must be prepared and compared against the original **MS** sample.

11.7.4. To determine the accuracy, calculate the percent recovery of the concentration for the analyte in the **MS**. Recovery must be within 80 – 120% of the true value.

11.7.5. To determine the precision, calculate the relative percent difference (RPD). The RPD must be <20% (**Section 13.3.4**).

11.8. Internal Standards

11.8.1. The internal standard listed in **Section 7.2** is added to all standards, QC samples, and samples.

11.8.2. The internal standard peak area for each injection is compared against the average peak area from the calibration curve.

11.8.3. The recovery of the internal standard must be 50 - 150% of the average peak area from the calibration curve.

11.8.4. If these criteria cannot be met (for example: a sample with a complex matrix), the data for such a sample must be reported with an appropriate qualifier, follow laboratory specific procedures.

12.0. Procedure

12.1. Sample Preparation:

12.1.1. Weigh 30.0 ± 3.0 mg of the material directly into a tared 2-mL centrifuge tube.

12.1.2. Spike 40.0 ng (10.0 µL, 4.00 µg/mL) **IWD** (**Section 8.4**).

12.1.3. Add 1000 µL methanol (**Section 8.2**)

12.1.4. Vortex until dissolved, or up to 30 seconds.

12.1.5. If not fully dissolved, centrifuge for 5 minutes at 12,000 RPM (13,362 × g).

12.2. Matrix Spike and Matrix Spike Duplicate Preparation (**MS and MSD**):

12.2.1. Weigh 30.0 ± 3.0 mg matrix (**Section 8.13**) directly into a tared 2-mL centrifuge tube.

12.2.2. Spike 10.0 ng (10.0 µL, 1000 ng/mL) working solution (**Section 8.9**).

12.2.3. Spike 40.0 ng (10.0 µL, 4.00 µg/mL) **IWD** (**Section 8.4**).

12.2.4. Add 1000 µL methanol (**Section 8.2**).

12.2.5. Vortex until dissolved, or up to 30 seconds.

12.2.6. If not fully dissolved, centrifuge for 5 minutes at 12,000 RPM (13,362 × g).

12.3. Laboratory Reagent Blank (**LRB**)

12.3.1. Label a 2-mL centrifuge tube.

12.3.2. Follow the steps in **Section 12.1.2** through **Section 12.1.5**.

12.4. Laboratory Fortified Blank (**LFB**)

12.4.1. Label a 2-mL centrifuge tube.

12.4.2. Follow the steps in **Section 12.2.2** through **Section 12.2.6.**

12.5. Matrix Blank (**MB**)

12.5.1. Label a 2-mL centrifuge tube

12.5.2. Follow the steps in **Section 12.2**, but omit instruction **12.2.2,** in which the Primary Working Solution of IBA and Azadirachtin is spiked. All other steps are identical.

12.6. LC-MS/MS Analytical Procedure.

12.6.1. Perform the initial LC-MS/MS calibration (**Section 10.1**) if needed.

12.6.2. Equilibrate the LC-MS/MS system with mobile phase flowing at the initial parameters described in **Table 5** in **Section 10.1.4**.

12.6.3. Analyze at least one **SBLK**, followed by a **LRB**. The **SBLK** must pass criteria in **Section 11.3.**

12.6.4. If it has been more than one month (>31 days) since running the last calibration curve, or if the mobile phase A was changed since the last curve, a new curve must be analyzed:

12.6.4.1. Analyze a full initial calibration (**Section 10.2**), followed by a **LRB** to assess potential carry-over.

12.6.4.2. Analyze a **CCR-WS** followed by a **LRB**.

12.6.4.3. The calibration curve and **CCR-WS** must pass the criteria set in **Section 10.3 and 10.4** respectively before samples can analyzed and reported.

12.6.5. If it has been one month or less (≤ 31 days) since running the last calibration curve and the mobile phase A has not been changed, the previous curve still needs to be verified as viable. If it fails verification then a new curve must be analyzed (see **Section 12.6.4**).

12.6.5.1. Analyze an **LOQ**, **CCV**, and **CCR-WS**, followed by a **LRB**.

12.6.5.2. The **LOQ**, **CCV**, and **CCR-WS** must pass criteria set in **Section 10.5.1** and Section **10.4.2** before any samples can be analyzed and reported.

12.6.6. Analyze up to 20 samples, including **LFB**, **MB**, **MS**, and **MSD** QC samples.

12.6.7. Analyze in the following order a **LRB**, **CCV**, and **LRB** at the end of the batch.

12.7. LC-MS/MS Integration/Quantitation Parameters

12.7.1. The integration of peaks is done by the software whenever possible. **Table 9** lists example parameters for integrating peaks using Analyst 1.6.1 software.

12.7.2. When the software inadequately integrates peaks, manual integration is necessary. A laboratory specific procedure must be available for manual peak integration.

Table 9: All analytes (Quantifying transitions only)

Parameters	**IBA (target analyte)**	**Azadirachtin (target analyte)**	**5-F-IBA (IS)**
Q1/Q3	202 / 116	719 / 659	220 / 134
Min. Peak Height	0	0	0
Min. Peak Width	0	0	0
RT window (sec)	10.0	10.0	30.0
Expected RT (min)	8.14	8.62	8.77
Smoothing Width	9	9	9
Use Relative RT	Yes	Yes	No
Automatic – IQAIII	No	No	No
Specify Parameters-MQ III	Yes	Yes	Yes
Noise Percent	50	50	50
Peak Splitting Factor	3	3	3
Base Sub Window (min)	0.4	0.4	0.4
Report Largest Peak	No	No	Yes

13.0. Data Acquisition, Reduction, Analysis, and Calculations

13.1. HPLC

13.1.1. **Table 5** (**Section 10.1.4**) summarizes the recommended operation conditions for the HPLC.

13.1.2. Calibrate or verify the calibration on each day of analysis as described in **Section 12.6.4** and **Section 12.6.5**. For routine analyses, the standards and sample extracts must be in extraction solvent (**Section 8.2**).

13.2. Identification of Analytes

13.2.1. Identify a sample component using relative retention time by comparing its retention time to the retention time of the IS. If the retention time of an unknown compound corresponds, within limits, to the relative retention time when compared to the IS, then initial identification is considered positive.

13.2.1.1. The width of the relative retention time window used to make identifications should be based upon measurements of actual retention time variations of standards and QC samples over the course of an analytical sequence. Three times the standard deviation of a retention time can be used to calculate a suggested window size for a compound. However, the experience of the analyst should weigh heavily in the interpretation of the chromatograms.

13.2.1.2. Current relative retention time windows are set at:

13.2.1.2.1. 0.920 – 0.940 for IBA

13.2.1.2.2. 0.970 – 0.990 for Azadirachtin

13.2.1.2.3. See **Section 10.1.4**, **Table 6** for additional retention time information.

13.2.2. Confirm a sample component after initial identification using the ratio of quantifying and qualifying peak areas.

13.2.2.1. The area ratio of quantifying to qualifying transitions used to make identifications should be based upon measurements of actual ratio variations over the course of multiple runs and concentration levels. Three times the standard deviation of a ratio can be used to calculate a suggested window size for a compound.

13.2.2.2. Current Quantifying/Qualifying ratios are listed in **Table 10**.

Table 10: Quantifying / Qualifying transition ion peak area ratios.

Analyte	Quantifying Transition (Da)	Qualifying Transition (Da)	Average Ratio	± 3 Standard Deviation	**Ratio Range
IBA	202 / 116	202 / 158	1.049	0.590	0.459 – 1.639
Azadirachtin	719 / 659	719 / 687	1.876	0.550	1.326 – 2.426

**Range in table serves as an example only. Ratio will be instrument dependent, the value should be based on the actual ratio of standards running on the instrument. Also, the ratio might change over time on the same instrument.

13.2.3. Identification requires expert judgment when sample components are not resolved chromatographically. When chromatographic peaks obviously represent more than one sample component (*i.e.,* broadened peak with shoulder(s) or a valley between two or more maxima), or any time doubt exists regarding the identification of a peak in a chromatogram, appropriate alternative techniques to help confirm peak identification are employed.

13.3. Calculations

13.3.1. Initial Calibration

13.3.1.1. Use the instrument software and specified parameters to perform peak integration for all identified peaks.

13.3.1.2. Calculate the relative retention time for each standard, by comparing the standard retention time to the equivalent **IS** retention time using the following formula. The relative retention time must pass the criteria set in

Section **13.2.1.2**. The calculation does not need to be done by hand if the software can calculate it.

$$Relative\ Retention\ Time = \frac{RT_{Std}}{RT_{IS}}$$

Where: RT_{Std} = retention time of the standard
RT_{IS} = retention time of the corresponding **IS**

For example, compare the RT of IBA to the RT of 5-F-IBA

13.3.1.3. Using the weighted 1/C linear regression curve for all calibration standards, check the curve linearity and calculate the standards recovery at each levels. Evaluate the linearity and recovery based on the criteria set in **Section 10.3**.

13.3.2. QC and unknown samples

13.3.2.1. Apply the linear regression calibration curve generated from the calibration standards to all QA/QC and unknown samples to calculate the concentration (ng/mL) of each analyte using the instrument quantification software.

13.3.2.2. For medical marijuana samples, this value must then be converted to a sample concentration in ng/g using the following equation:

$$C_s\left(\frac{ng}{g}\right) or\ (ppb) = \frac{C_E\left(\frac{ng}{mL}\right) * V_F(mL) * D}{M_I(mg) * 0.001(\frac{g}{mg})}$$

Where:

C_S = Concentration of analyte in sample (ng/g) or (ppb)
C_E = Concentration of analyte in solvent (ng/mL) (from software)
V_F = Final volume of extract (mL)
M_I = Initial mass of sample (mg)
0.001 g/mg = Conversion from mg to g
D = Dilution factor, if applicable.

13.3.3. Matrix Spike and Matrix Spike Duplicate (**MS** and **MSD**)

13.3.3.1. To determine the precision, calculate the relative percent difference (RPD). The RPD must be <20%.

$$RPD = \frac{|MS - MSD|}{\left(\frac{|MS + MSD|}{2}\right)} * 100$$

Where: RPD is in percent (%).
MS = Matrix Spike concentration in ppb.
MSD = Matrix Spike Duplicate concentration in ppb.

13.3.4. Calculate the average **IS** peak area from the calibration curve. Evaluate the system stability by using the following equation on every injection, and comparing to the criteria set in **Section 11.8**.

$$IS\ Peak\ Area\ Deviation\ (\%) = \frac{|IS_I - IS_A|}{IS_A} * 100$$

Where: IS_I = IS peak area for individual injection
IS_A = IS peak area average from calibration curve

13.3.5. Do not use daily calibration verification standards to calculate the concentration of analytes in samples.

13.4. Reporting of Results

13.4.1. Non-detected analyte(s) are reported as less than (<) the **LOD** as specified in **Section 1.1**.

13.4.2. Analyte(s) detected at a concentration at or above the **LOQ** are reported using 2 significant figures.

13.4.3. Analyte(s) detected at a concentration at or above the **LOD**, but below the **LOQ** are reported as less than (<) the **LOQ** as specified in **Section 1.1**.

13.4.4. Analyte(s) detected at a concentration below the **LOD** are considered non-detects due to the uncertainty of the actual presence of the analyte and are reported as less than (<) the **LOD** as specified in **Section 1.1**.

13.4.5. Analytes detected at a concentration above the **ULOQ** cannot be accurately reported. A new sample must be prepared using a smaller amount of sample or dilute the current sample if there has no more extra sample available to use. Use the approximate concentration to adjust the sample size. If there is not enough for a new preparation, report as greater than (>) the **ULOQ**.

14.0. Data Assessment, Acceptance Criteria, and Corrective Actions for Out-of-Control Data

14.1. All analytical batches must meet all quality control criteria as described within this procedure and all quality control results must be documented.

14.2. The acceptance criteria for standards and QC samples are defined in **Section 10**, and **Section 11**. The sections below (**Sections 14.3 – 14.14**) outline the most common corrective action procedures for nonconforming data and inconsistent chromatograms. Since re-injection of a standard or sample is a routine corrective action for most nonconformities, it is not included in each individual section below, but may be used whenever applicable.

14.3. Failure to meet QC criteria for a Calibration Curve Correlation Factor of ≥0.995.

14.3.1. Assess the calibration curve to determine if there is one particular standard that appears to be prepared incorrectly. If so, re-prepare that standard and analyze. If more than eight (8)

hours has elapsed since the original failing calibration standard was analyzed, then all calibration standards must be re-analyzed.

14.3.2. If more than one standard appears to be prepared incorrectly, or the calibration curve is erratic, re-prepare all calibration standards and analyze. This may involve re-preparing the working standard solution or opening new stock standard solutions.

14.3.3. If necessary, perform instrument maintenance.

14.3.4. A correlation factor of ≥0.995 must be achieved before sample analysis can begin. If samples were analyzed before an acceptable calibration curve was established, all affected samples must be re-analyzed under an acceptable curve or the results will be appropriately qualified.

14.4. Cross Check Reference Standard (**CCR**) failure to meet the 80-120% recovery criteria.

14.4.1. Check the calibration curve linearity (**Section 10.3.3**), calibration curve response (**Section 10.3.4**), and internal standards response (**Section 11.8**).

14.4.2. Check **LFB** recovery value (**Section 11.5**).

14.4.3. If the **LFB** and other responses of standards curve appear normal, then the current **CCR** is likely compromised and a new **CCR** will be prepared and re-analyzed; it may be necessary to prepare a new stock solution or working solution.

14.4.4. If the criteria fail for **LFB** recovery, and/or the curve linearity and/or the curve response, the calibration curve is likely compromised, and a new curve will need to be made.

14.4.4.1. If the curve that failed comes from a freshly prepared **PS** or **WS**, one or both may need to be remade.

14.5. Failure to meet required QC criteria for Continuing Calibration Check standard (**CCV**) of 80% to 120% recovery.

14.5.1. A new **CCV** is prepared and re-analyzed; it may be necessary to prepare from new working or stock solutions.

14.5.2. If a newly prepared **CCV** still does not meet the required criteria, the instrument is recalibrated with new calibration standards, which may be prepared from new or existing working standard solutions or stock standard solutions. A new calibration curve is prepared and analyzed on the instrument, and is verified with a **CCR** using the new curve.

14.5.3. All samples must be bracketed by an acceptable **CCV**. Any samples that are analyzed without an acceptable bracket must be reanalyzed when an acceptable **CCV** is achieved or a new calibration is established. If reanalysis in not possible due to lack of remaining extract or sample, the original sample results will be appropriately qualified.

14.6. Failure to meet required QC criteria for **LOQ** of 70% to 130% recovery.

14.6.1. A new **CalS-1b** sample is prepared and re-analyzed; it may be necessary to prepare this from new working or stock solutions.

14.6.2. If a newly prepared **CalS-1b** sample still does not meet the required criteria. The instrument is recalibrated with new calibration standards, which may be prepared from new or existing working standard solutions or stock standard solutions. A new calibration curve is prepared and run on the instrument, and verified with a **CCR** using the new curve.

14.6.3. A **CalS-1b** sample within 70-130% recovery must be achieved before quantification analysis can begin. If samples were analyzed before an acceptable **LOQ** was achieved, all affected samples must be re-analyzed after an acceptable **LOQ** is achieved.

14.7. Failure to meet required QC criteria for System Blank of <1/3 **LOQ** for target analyte(s).

14.7.1. Replace the mobile phase with freshly made mobile phase.

14.7.2. Change the pre-column.

14.7.3. Clean the column at an appropriate temperature by extended flow of a strong solvent such as isopropanol until such time as contaminants are removed from the column based on column manufacturer's recommendations.

14.7.4. Inject multiple system blanks and run them through the system until background contamination is removed or reduced to an acceptable level.

14.7.5. An acceptable system blank must be achieved before sample analysis begins. If samples have already been analyzed, then any samples containing target analytes must be re-analyzed. If re-analysis of suspect samples is not possible due to lack of remaining extract or sample, the original sample results will be appropriately qualified.

Exception: If the samples do not contain target analytes at or above the **LOQ**, the original results may be reported without re-analysis and qualification is not necessary.

14.8. Failure to meet required QC criteria for Laboratory Reagent Blank of <1/3 **LOQ** for target analyte(s).

14.8.1. Analyze a system blank to ensure that the system is free from background contamination. If background contamination is discovered in the system blank, follow the corrective actions described above (**Section 14.7**)

14.8.2. Re-inject the **LRB** once a contaminant-free system is achieved.

14.8.3. If the re-injection still fails, request that a new **LRB** be extracted and analyze to ensure that a systemic problem does not exist. If a new **LRB** has already been extracted with a subsequent batch then the extraction of an additional **LRB** is not required.

14.8.4. An acceptable **LRB** must be achieved before sample analysis begins. If samples have already been analyzed, then any samples containing target analytes must be re-analyzed (if system contamination is suspected) or re-extracted and analyzed (if extraction contamination is suspected). If re-analysis or re-extraction of suspect samples is not possible due to lack of remaining extract or sample, the original sample results will be appropriately qualified.

Exception: If the samples do not contain target analytes at or above the **LOQ**, the original results may be reported without re-analysis and qualification is not necessary.

14.8.5. If subsequent **LRB**s continue to show unacceptable levels of background contamination, the extraction of additional samples must be halted until the source of the contamination can be determined and eliminated or reduced to acceptable levels.

14.9. Failure to meet required QC criteria for MB of <1/3 LOQ for target analyte(s).

14.9.1. Analyze MB to ensure that the system is free from background contamination. If background contamination is discovered in the system blank, follow the corrective actions described above (Section **14.7**)

14.9.2. Re-inject the MB once a contaminant-free system is achieved.

14.9.3. If the re-injection still fails, request that a new MB be extracted and analyze to ensure that a systemic problem does not exist. If a new MB has already been extracted with a subsequent batch then the extraction of an additional blank is not required.

14.9.4. An acceptable MB must be achieved before sample analysis begins. If samples have already been analyzed, then any samples containing target analytes must be re-analyzed (if system contamination is suspected) or re-extracted and analyzed (if extraction contamination is suspected).

Exception - If the samples do not contain target analytes at or above the LOQ, the original results may be reported without re-analysis and qualification is not necessary.

14.9.5. If subsequent MBs continue to show unacceptable levels of background contamination, the extraction of additional samples must be halted until the source of the contamination can be determined and eliminated or reduced to acceptable levels.

14.10. Failure to meet required QC criteria for Laboratory Fortified Blank (**LFB**) as described in **Section 11.5**.

14.10.1. Check to determine whether there is an interference peak that was not identified.

14.10.2. Reanalyze the **LFB** sample. If it is still out of the range, check the **MS** for a similar problem.

14.10.3. If the **MS** also fails, the problem is likely to be related to the spiking solution. Discard the problematic solution and re-prepare the spiking solution, **LFB**, and **MS**.

14.10.4. If the **MS** passes, there is no problem with the spiking solution, but the **LFB** must be re-prepared.

14.11. Failure to meet required QC accuracy (recovery) criteria for Matrix Spike (**MS**) as described in **Section 11.6.3**.

14.11.1. Check the **LFB** recovery to see if it is related to the spiking solution.

14.11.2. If the **LFB** also fails, discard problematic spiking solution and re-prepare the spiking solution, **LFB**, **MS,** and **MSD**.

14.11.3. If the subsequent **MS** is prepared with a newly prepared spiking solution and meets acceptance criteria, no further action is required.

14.11.4. If the **MS** fails to meet the acceptance criteria, but the **LFB** is acceptable, then it is recommended that the **MS** and/or **MSD** be re-prepared and analyzed if sufficient sample remains; this may require preparation from a new stock standard.

14.11.5. If re-analysis is performed and the new **MS** and/or **MSD** meets the acceptance criteria, only report those results.

14.11.6. If the **MS** and/or **MSD** cannot be reanalyzed, or if the re-analyzed **MS** and/or **MSD** still fails, the original **MS** and/or **MSD** and all corresponding sample results will be appropriately qualified on the report.

14.12. Failure to meet required QC precision (RPD) criteria for Matrix Spike as described in **Section 11.7.**

14.12.1. Compare the **MS** and **MSD** for **IS** Peak Area Deviation (**Section 13.3.4**) using only the **IS** peak area from these two samples.

14.12.2. If the **IS** deviation check fails, both **MS** and **MSD** must be re-prepared, because the **IS** was not accurately spiked in.

14.12.3. If the deviation check passes, repeat the calculation using the peak areas of both samples in place of **IS** peak area.

14.12.4. If this deviation check fails, both **MS** and **MSD** must be re-prepared, because the analyte spike was not accurately spiked in.

14.12.5. If samples do not deviate from peak area or **IS** peak area, yet still fail precision criteria, both **MS** and **MSD** must be re-prepared.

14.12.6. If the re-prepared **MS** and/or **MSD** still fail RPD, the original **MS** and/or **MSD** and all corresponding sample results will be appropriately qualified on the report.

14.13. Failure to meet required QC criteria for IS peak area variation described in **Section 11.8**.

14.13.1. See **Section 11.8.4**.

14.14. Inconsistent baseline

14.14.1. Replace the mobile phase with freshly made mobile phase.

14.14.2. Perform appropriate instrument maintenance, if applicable.

14.14.3. Repeat the sequence using the same standards/samples. If repeat analysis is acceptable, report only those results.

14.14.4. If instrument maintenance and repeat analysis fails to produce acceptable data, the sample results will be appropriately qualified.

14.15. All other nonconforming data that has not been addressed within this procedure requires the completion of a nonconformance/corrective action report.

15.0. Method Performance

15.1. Detection limit study results and demonstration of capability study results are maintained by the laboratory.

16.0. Waste Management/Pollution Prevention

16.1. It is the responsibility of the laboratory to comply with all federal, state and local regulations governing waste management, particularly with regard to hazardous waste identification rules and land disposal restrictions.

16.2. Minimize solvent, chemical, reagent, and standard use whenever possible to reduce the amount of hazardous waste generated.

16.3. Dispose of solvent waste in an appropriate solvent in a properly labeled waste container.

16.3.1. Acetonitrile and methanol must be disposed of separately from all other solvents in a container no larger than 4 L (1-Gallon).

16.3.2. All other solvents are separated into two categories: chlorinated and non-chlorinated. These are disposed of in red, 5-Gallon solvent cans.

16.4. Dispose of non-hazardous aqueous waste in the laboratory sink followed by flushing with tap water.

16.5. Dispose of glassware in appropriately labeled boxes. Be sure that, whenever possible, the glass has been thoroughly rinsed and is contaminant-free before disposal.

17.0. References

17.1. AB Sciex 4500 Series of Instruments – System User Guide http://sciex.com/Documents/Downloads/Literature/4500-system-user-guide-en.pdf

17.2. Shimadzu HPLC Module Manuals, http://store.shimadzu.com/s-1003-liquid-chromatography.aspx?pagenum=1

17.3. Pan, X. & Wang, X. Profiling of plant hormones by mass spectrometry. J. Chromatogr. B 877, 2806-2813 (2009).

17.4. Public Health Law, section 502 of the Public Health Law (PHL), Title 10 (Health) of The Official Compilation of Codes, Rules and Regulations of the State of New York (NYCRR) subpart 55-2 (Approval of Laboratories Performing Environmental Analysis). http://w3.health.state.ny.us/dbspace/NYCRR10.nsf/56cf2e25d626f9f785256538006c3ed7/c9252587bc832b3485256c390055920a?OpenDocument&Highlight=0,section,55)

17.5. Definition and Procedure for the Determination of the Method Detection Limit, Environmental Protection Agency, 40 CFR Part 136, Appendix B.