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Determination of the Plant Growth Regulator Indole-3-butyric Acid and Pesticides using LC-MS/MS
NYS DOH MML-306

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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] (ppb) In Olive Oil Matrix	LLOQ[2] (ppb) In Olive Oil Matrix	LOD (ppb) In Tincture Matrix	LLOQ (ppb) In Tincture Matrix
Indole-3-butyric acid (IBA)	133-32-4	3.2	16	1.6	10
Azadirachtin	11141-17-6	0.9	10	1.9	10
5-Fluoroindole-3-butyric acid (5-FIBA, Internal Standard)	319-72-2	N/A	N/A	N/A	N/A

[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] Lower Limit of Quantitation (LLOQ) – 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**.

2.0. Summary of the Method

2.1. After liquid extraction with an aqueous-organic solvent mixture, 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 detectection of the analyte and IS. Chromatography of IBA 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. The limits of detection (LOD) for IBA and Azadirachtin in an olive oil matrix is 3.2 ppb and 0.9 ppb, and the lower limits of quantification (LLOQ) is 16 ppb and 10 ppb. The limits of detection (LOD) for IBA and Azadirachtin in a tincture matrix is 1.6 ppb and 1.9 ppb, and the lower limits of quantification (LLOQ) is 10 ppb and 10 ppb.

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 extraction solvent that is spiked with known quantities of target analytes and and prepared and analyzed as if it were a sample. The **LFB** is used to evaluate the accuracy of the methodology. 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 matrix that is spiked with known quantities of target analytes and extracted/analyzed as a quantifiable sample. The matrix from which the portion to be spiked was taken must be analyzed separately to determine the levels of background analyte concentrations. The **MS** is used to correct for background concentrations of the analyte and to determine whether the sample matrix contributes bias to the sample results. The **MS** is also used to determine the accuracy of the methodology in a manner similar to that of the **LFB**. 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. Acronym: Method Detection Limit (MDL).

3.16. Lower Limit of Quantitation (**LLOQ)** – 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 **LLOQ** for the analyte. The **LLOQ** is at least 5 times the **LOD**. The LLOQ value also must meet the 70-130% recovery limitation and the %RSD must be <30%. If it is does not, the LLOQ must be raised to the point where these requirements are met.

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. Sample Batch **–** A group of samples that are processed together as a unit using the same procedure and materials. A typical batch consists of 20 samples. A batch includes the necessary quality controls, including method blanks, laboratory fortified blanks, matrix spikes, duplicates, and samples.

4.0. Health and Safety Warnings

4.1. Caution must be used when working with IBA, Azadirachtin, and 5-FIBA. According to the Material Safety Data Sheets (MSDSs), 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 MSDSs for proper storage, handling, and disposal of solvents, reagents, and standards. MSDSs 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 in 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. Syringes, various sizes.

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

6.1.3. Disposable pipettes.

6.1.4. Pipette bulbs.

6.1.5. 2-ml auto-sampler vials with 0.3-ml target poly-spring inserts and Teflon-lined screw-caps or crimp-top caps.

6.1.6. Micro pipette controller, various sizes, Eppedorf, Research Plus, or equivalent.

6.2. Sample Extraction Equipment

6.2.1. Analytical balance, Mettler-Toledo, model # XSE205DU, or equivalent (DEHS-005-SOP).

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 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 Q-trap 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. Hemp oil – (Hemp Meds, Real Scientific Hemp Oil, Gold Grade or equivalent).

7.1.6. Olive oil – (Sigma-Aldrich, Catalog # 01514-500mL, or equivalent).

7.1.7. Ethanol – (200 proof, Pharmco-AAPER, Catalog # 111000200, or equivalent).

7.1.8. Propylene Glycol – (Baker, Catalog # U510-07, or equivalent).

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

7.1.10. Acetone – (Mallinckrodt, Catalog # 2432, 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 possible.

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 #	Solvent
Indole-3-butyric acid (IBA)	Sigma-Aldrich	45532-250MG	Solid
Azadirachtin	Sigma-Aldrich	A7430-17MG	Solid
5-Fluoroindole-3-butyric acid (5-FIBA, IS)	Oakwood Chemical	040689-1G	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. Add 80 mL of HPLC-grade water to a 200-mL volumetric flask.

8.2.2. Dilute to volume with methanol; invert 7x to mix.

8.2.3. Dilute to volume again; invert 7x to mix.

8.2.4. Solution is stable for 6 months at -20 °C, or 1 month at room temperature.

8.3. Internal Standard (**IS**) – @1 mg/mL for 5-FIBA.

8.3.1. Take an accurately weighed 10 mg (± 0.1 mg) reference standard of 5-FIBA acid in a calibrated 10-mL volumetric flask (certified "A" class).

8.3.2. Fill to volume with methanol and invert 3x to mix.

8.3.3. Each **IS** solution is filled and sealed in a 1.5-mL crimp-cap amber vial and labeled according to LOAC-029-SOP. Store the vials in freezer at -20 °C.

8.3.4. Store at -20 °C for up to 6 months, in a sealed vial or ampule.

8.4. Internal Standard Stock Diluent **(ISD)**

8.4.1. Fill a 1.0-mL volumetric flask 3/4 full with extraction solvent (**section 8.2**).

8.4.2. Add 100 µL of **IS** 5-FIBA @ 1 mg/mL.

8.4.3. Dilute to volume with extraction solvent and invert 7x to mix.

(1 mg/mL) (10 µL) = (X µg/mL) (1000 µL)

X= 100 µg/mL

8.4.4. 1.0 mL of the solution is transferred and sealed in a 1.5 mL crimp-cap vial and labeled according to LOAC-029-SOP. Store the vials in a freezer at -20 °C.

8.4.5. Store at -20 °C for up to 6 months, in a sealed vial or ampule.

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

8.5.1. Fill a 1.0-mL volumetric flask 1/2 full with extraction solvent (**section 8.2**).

8.5.2. Add 400 µL of **ISD** @ 100 µg/mL.

8.5.3. Dilute to volume with extraction solvent and invert 7x to mix.

(100 µg/mL) (400 µL) = (X µg/mL) (1000 µL)

X= 40 µg/mL

8.5.4. Solution is transferred and sealed in 1.5 mL crimp-cap vial and labeled according to LOAC-029-SOP. Store the ampules in freezer at -20 °C.

8.5.5. Store at -20 °C for up to 6 months, in a sealed vial or ampule.
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8.6. Primary Stock Standards (**PS**) – @ 1 mg/mL for IBA.

8.6.1. **PS** is only used when preparing a calibration curve.

8.6.2. Take an accurately weighed 10 mg (± 0.1 mg) reference standard of IBA in a calibrated 10 mL volumetric flask (certified "A" class).

8.6.3. Fill to volume with methanol and invert 3X to mix.

8.6.4. The **PS** solution is filled and sealed in a 1.5-mL crimp-cap amber vial and labeled according to LOAC-029-SOP. Store the vial in a -20 °C freezer.

8.6.5. Store at -20 °C for up to 6 months, in a sealed vial or ampule.

8.7. Secondary Stock Standard – @ 1 mg/mL for IBA.

8.7.1. Take an accurately weighed 10 mg (± 0.1 mg) reference standard of each analyte in a calibrated 10 mL volumetric flask (certified "A" class).

8.7.2. Fill to volume with methanol and invert 3x to mix.

8.7.3. This solution is transferred and sealed in a 1.5-mL crimp-cap amber vial and labeled according to LOAC-029-SOP. Store the vial in a freezer at -20 °C.

8.7.4. Store at -20 °C for up to 6 months, in a sealed vial or ampule.

8.8. Primary Stock Standards (**PS**) – @ 1 mg/mL for Azadirachtin.

8.8.1. **PS** is only used when preparing a calibration curve.

8.8.2. Take an accurately weighed 10 mg (± 0.1 mg) reference standard of Azadirachtin in a calibrated 10 mL volumetric flask (certified "A" class).

8.8.3. Fill to volume with methanol and invert 3X to mix.

8.8.4. The **PS** solution is filled and sealed in a 1.5-mL crimp-cap amber vial and labeled according to LOAC-029-SOP. Store the vial in a -20 °C freezer.

8.8.5. Store at -20 °C for up to 6 months, in a sealed vial or ampule.

8.9. Secondary Stock Standard – @ 0.5 mg/mL for Azadirachtin.

8.9.1. Take an accurately weighed 5 mg (± 0.05 mg) reference standard of each analyte in a calibrated 10 mL volumetric flask (certified "A" class).

8.9.2. Fill to volume with methanol and invert 3x to mix.

8.9.3. This solution is transferred and sealed in a 1.5-mL crimp-cap amber vial and labeled according to LOAC-029-SOP. Store the vial in a freezer at -20 °C.

8.9.4. Store at -20 °C for up to 6 months, in a sealed vial or ampule.

8.10. Primary Working Standard (**WS**) – @ 10 µg/mL for IBA and Azadirachtin.

8.10.1. **WS** is only used when preparing a calibration curve.

8.10.2. Fill a 1.00-mL volumetric flask 1/2 full with extraction solvent solution (**Section 8.2**).

8.10.3. Add 10 µL of IBA **PS** @ 1mg/mL (**Section 8.6**) and 10 µL of Azadirachtin **PS** @ 1mg/mL (**Section 8.8**).

8.10.4. Dilute to volume with extraction solvent solution and invert 3x to mix.

$$(1 \text{ mg/mL})\ (X \text{ mL}) = (10 \text{ µg/mL})\ (1.00 \text{ mL})$$

$$X = 0.01 \text{ mL} = 10 \text{ µL}$$

8.10.5. This solution is filled and sealed in a 1.5-mL crimp-cap amber vial and labeled according to LOAC-029-SOP. Store the vial in a -20 °C freezer.

8.10.6. Store at -20 °C for up to 6 months, in a sealed vial or ampule.

8.11. Secondary Working Standard – @ 10 µg/mL for IBA and Azadirachtin.

8.11.1. Fill a 1.00-mL volumetric flask 1/2 full with extraction solvent solution (**Section 8.2**).

8.11.2. Add 10 µL of IBA **Secondary Stock Standard** @ 1mg/mL (**Section 8.7**) and 20 µL of Azadirachtin **Secondary Stock Standard** @ 0.5 mg/mL (**Section 8.9**).

8.11.3. Dilute to volume with extraction solvent solution and invert 3x to mix.

$$(1 \text{ mg/mL})\ (X \text{ mL}) = (10 \text{ µg/mL})\ (1.00 \text{ mL})$$

$$X = 0.01 \text{ mL} = 10 \text{ µL}$$

$$(0.5 \text{ mg/mL})\ (X \text{ mL}) = (10 \text{ µg/mL})\ (1.00 \text{ mL})$$

$$X = 0.02 \text{ mL} = 20 \text{ µL}$$

8.11.4. This solution is filled and sealed in a 1.5-mL crimp-cap amber vial and labeled according to LOAC-029-SOP. Store the vial in a -20 °C freezer.

8.11.5. Store at -20 °C for up to 6 months, in a sealed vial or ampule.

8.12. Cross check reference standard working solution (CCR-WS) @ 100 ng/ml. The CCR-WS is prepared as described below. The CCR-WS is prepared at a concentration within the middle of the calibration curve.

8.12.1. Fill a 1.00 mL volumetric flask 1/2 full with extraction solvent solution (**Section 8.2**).

8.12.2. Add 10 µL of Secondary Working Standard @ 10 µg/ml (**Section 8.11**).

8.12.3. Dilute to volume with extraction solvent solution and invert 3x to mix.

8.12.4. This solution is transferred and sealed in a 1.5 mL crimp-cap amber vial and labeled according to LOAC-029-SOP. Store the vial in freezer at -20 °C.

8.12.5. Store at -20 °C for up to 6 months, in a sealed vial or ampule.

8.13. Mobile Phases

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

8.13.1.1. 0.315 g of ammonium formate is added to a scintillation vial and diluted with a small volume of HPLC-grade water.

8.13.1.2. Ammonium formate solution prepared in section 8.10.1.1 is transferred to a 1-L volumetric flask. The scintillation vial is rinsed well with HPLC-grade

water, and 0.2 mL of formic acid is added to the 1-L volumetric flask. Dilute to volume with HPLC-grade water and mix well.

8.13.1.3. This solution must be prepared monthly.

8.13.2. Mobile phase B: Methanol.

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

8.14. Matrices

8.14.1. Olive Oil Matrix (stored at -20 °C for up to 6 months)

8.14.1.1. Weigh 2000 ± 200 mg of hemp oil extract into a 15-mL centrifuge tube.

8.14.1.1.1. Hemp oil – Hemp Meds, Real Scientific Hemp Oil, Gold Grade or equivalent.

8.14.1.2. Add 8000 ± 800 mg olive oil.

8.14.1.2.1. Olive oil – Sigma-Aldrich, Catalog # 01514-500mL, or equivalent.

8.14.1.3. Sonicate for 5 minutes.

8.14.1.4. Vortex for 1 minute.

8.14.1.5. Sonicate for an additional 5 minutes.

8.14.2. Tincture Matrix (stored at -20 °C for up to 6 months)

8.14.2.1. Weigh 100 ± 10 mg of hemp oil extract into 15-mL centrifuge tube.

8.14.2.1.1. Hemp oil – Hemp Meds, Real Scientific Hemp Oil, Gold Grade or equivalent.

8.14.2.2. Add 5000 ± 500 mg propylene glycol.

8.14.2.2.1. Propylene glycol – Baker, Catalog # U510-07, or equivalent.

8.14.2.3. Add 5000 ± 500 mg ethanol.

8.14.2.3.1. Ethanol, 200 proof – Pharmco-AAPER, Catalog # 111000200, or equivalent.

8.14.2.4. Sonicate for 5 minutes.

8.14.2.5. Vortex for 1 minute.

8.14.2.6. Sonicate for an additional 5 minutes.

9.0. Sample Transport, Receipt, Preservation, Handling, 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. MM products from the ROs are received into the Wadsworth Center as per the Receipt of Medical Marijuana Product from the Registered Organization SOP (see MML-100-SOP).

9.2.2. Samples received into the Wadsworth Center from the ROs must use the NYSDOH Request for Analysis form (DOH-246 RFA).

9.2.3. 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. Sample Analysis:

9.4.1. Samples for analysis are placed in the autosampler, which is set to a temperature of 4 °C.

9.5. Extract Storage:

9.5.1. 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 calibrated twice per year using 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. The current optimal MS/MS conditions are described in **Tables 3** and **4**.

Table 3: Instrument specific MS/MS parameters:

Parameter	Value
MS Acquisition Time:	7 – 11.0 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 °C
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)	Scheduled MRM analysis window (min)
IBA	Negative	202	**116** (158)	**-25** (-17)	7.62 – 9.62
Azadirachtin	Negative	719	**659** (687)	**(-20)** (-15)	9.50 – 9.70
5-FIBA, (IS)	Negative	220	**134** (147)	**-50** (-18)	8.65 – 10.65

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

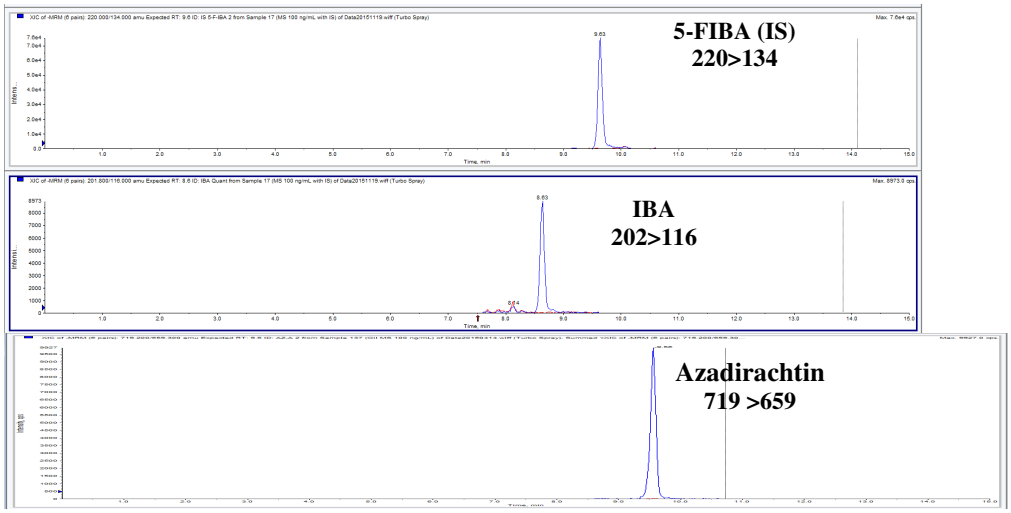
Column	Value
Column:	Agilent Poroshell 120 EC-C18, 2.7 µm, 3.0 x 150 mm, # 693975-302
Guard Column:	Phenomenex SecurityGuard, Analytical Guard Cartridge System; # KJ0-4282
Column Temperature:	40.0 °C
Autosampler	**Value**
Temperature:	4.0 °C
Injection Volume:	10.0 µL
Injection Loop Volume:	50.0 µL
Rinse 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)
6.0 min:	Divert to MS detector (Position B)
10.0 min:	Divert to waste (Position A)
HPLC Gradient, Time	**Percentage of Mobile Phase B**
Initial:	10%
2.0 min:	50%
10.0 min:	58%
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	8.63	8.53 – 8.73
Azadirachtin	9.60	9.50 – 9.70
5-FIBA (IS)	9.63	9.53 – 9.73

Figure 1. Chromatogram of the medium level of spiked extraction (100 ng/mL IBA and Azadirachtin spike) in olive oil matrix. The 100 ng/mL, equivalent to 200 ppb IBA and Azadirachtin spike in olive oil matrix (blue), is shown overlaid on the chromatogram recorded for the olive oil matrix blank (red).



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

10.2.1. Serial dilutions are made from the Primary working solution of IBA and Azadirachtin (**Section 8.10**). (The concentration range is 50 ng/mL to 10000 ng/mL.) (see Table 7)

10.2.1.1. ***CalS 6a*** (10000 ng/mL)

10.2.1.2. CalS 6a is the same as the Primary Working Standard (Section 8.10).

10.2.2. ***CalS 5a*** (5000 ng/mL)

10.2.2.1. Dispense 500 µL of CalS6a (**Section 10.2.1**) into 2-mL autosampler vial with a Teflon-lined screw cap, labeled **CalS 5a,** containing 500 µL of extraction solvent.

10.2.2.2. Mix well inverting the autosampler vial, at least 10 times.

(10000 ng/mL) (500 µL) = (X ng/mL) (1000 µL)

X= 5000 ng/mL

10.2.3. ***CalS 4a*** (1000 ng/mL)

10.2.3.1. Dispense 200 µL of **CalS 5**a (**Section 10.2.2**) into a 2-mL autosampler vial with a Teflon-lined screw cap, labeled **CalS 4a,** containing 800 µL of extraction solvent.

10.2.3.2. Mix well using auto sampler vial, at least 10 times.

(5000 ng/mL) (200 µL) = (X ng/mL) (1000 µL)

X= 1000 ng/mL

10.2.4. ***CalS 3a*** (500 ng/mL)

10.2.4.1. Dispense 500 µL of **CalS 4a** (**Section 10.2.3**) into into a 2-mL autosampler vial with a Teflon-lined screw cap, labeled **CalS 3a,** containing 500 µL of extraction solvent.

10.2.4.2. Mix well using autosampler vial, at least 10 times.

(1000 ng/mL) (500 µL) = (X ng/mL) (100 µL)

X= 500 ng/mL

10.2.5. ***CalS 2a*** (100 ng/mL)

10.2.5.1. Dispense 200 µL of **CalS 3a** (**Section 10.2.4**) into a 2-mL autosampler vial with a Teflon-lined screw cap, labeled **CalS 2a,** containing 800 µL of extraction solvent.

10.2.5.2. Mix well using auto sampler vial, at least 10 times.

(500 ng/mL) (20 µL) = (X ng/mL) (1000 µL)

X= 100 ng/mL

10.2.6. ***CalS 1a*** (50 ng/mL)

10.2.6.1. Dispense 500 µL of **CalS 2a** (**Section 10.2.5**) into 2-mL autosampler vial with a Teflon-lined screw cap labeled **CalS 1a** containing 500 µL of extraction solvent.

10.2.6.2. Mix well inverting the autosampler vial, at least 10 times.

(100 ng/mL) (500 µL) = (X ng/mL) (1000 µL)

X= 50 ng/mL

Table 7. – Calibration Curve without IS

CalS STD	Std Conc. (ng/mL)	Std Ref Section ID	Volume of IBA STD	Extraction Solvent (Section 8.2)
CalS 6a	10000	10.2.1	-	-
CalS 5a	5000	10.2.2	500 µL	500 µL
CalS 4a	1000	10.2.3	200 µL	800 µL
CalS 3a	500	10.2.4	500 µL	500 µL
CalS 2a	100	10.2.5	200 µL	800 µL
CalS 1a	50	10.2.6	500 µL	500 µL

10.2.7. Prepare calibration standard mixtures with **IWD** by diluting 100 µL of each **CalS** (**Section 10.2.1 – 10.2.6**) with 10 µL of **IWD** @ 4 µg/mL (**Section 8.5**) and 900 µL extraction solvent (**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 can be seen 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	Extraction Solvent (Section 8.2)	IS Final Concentration (ng/mL)
CalS 6b	1000	10.2.1	100 µL	8.5	10 µL	900 µL	400
CalS 5b	500	10.2.2	100 µL	8.5	10 µL	900 µL	400
CalS 4b	100	10.2.3	100 µL	8.5	10 µL	900 µL	400
CalS 3b	50	10.2.4	100 µL	8.5	10 µL	900 µL	400
CalS 2b	10	10.2.5	100 µL	8.5	10 µL	900 µL	400

CalS 1b	5	10.2.6	100 µL	8.5	10 µL	900 µL	400

10.2.8. 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/C 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 the analyte.

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

10.3.3. The absolute **IS** response in each chromatographic run must not deviate by more than 10% from its average value for each matrix analyzed.

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

10.3.5. 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.

10.3.6. If all of these 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 working solution (**CCR-WS**) @ 100 ng/mL.

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

10.5. Continuing Calibration Check

10.5.1. The calibration curve and average response factors must be verified on each working day by the measurement of a low limit of quantitation (**LLOQ**) standard and a minimum of two continuing calibration verification standards (**CCV**), one at the beginning and one at the end of the sample batch. The beginning **CCV** and **LLOQ** standard may be substituted by a full initial calibration. For extended periods of analysis (greater than 8 h), it is strongly recommended that **CCV**s be interspersed with samples at regular intervals during the course of the analysis at varying concentrations. The response for any analyte in the **LLOQ** standard must be within ± 30% of the predicted response. The **CCVs** must be within ± 20% of the predicted response for routine sample batches.

10.5.2. The **CCV** and **LLOQ** standards are prepared at the following concentrations but higher or lower levels may be prepared as necessary. The **CCV** standard must be at a concentration within the calibration curve and the **LLOQ** standard must be at a concentration at or below the **LLOQ** as listed in (Section 1.1).

10.5.2.1. Continuing Calibration Checks (CCVs) are prepared at the following levels:

10.5.2.1.1. 10 ng/mL, 50 ng/mL, 100 ng/mL, and 500 ng/mL (same as **CalS 2b** thru **5b** – (**Section 10.2 Table 8**)

10.5.2.2. LLOQ standard for IBA and Azadirachtin is at 5 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 analyte. The initial DOC must consist of the analysis of four or five matrix spike samples that have been fortified with the analyte at a mid-level concentration (50-100 ng/mL). 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. For the analyte, the recovery value for all replicates must fall in the range of ± 20%. 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. For the analyte, the recovery value for all replicates must fall in the range of ± 20%. The precision of the measurements, calculated as relative standard deviation (RSD), must be 20% or less. When analyses fail to meet that fail 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, such as an external proficiency test (PT) sample.

11.1.1.2.2. Internally prepared PT sample in which all analytes concentration have been accurately measured.

11.1.1.2.3. Acceptable performance of an initial DOC as described above in 11.1.1.

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 11.6** for routine samples or the secondary source standard per **Section 10.4**.

11.2. Method Detection Limits

11.2.1. An **LOD** study must be completed for the target analyte as indicated in **Section 1.1**, on each instrument used to analyze sample extracts, and in each sample matrix. An **LOD** study consists of the analysis of seven to ten low level matrix spike samples that have been fortified with the analyte at no more than twice the laboratory's minimum reporting limit. The fortified samples must be treated as if they were real samples and processed through all of the applicable method procedures (**Section 12.1**). It is recommended that the low-level matrix spike samples be prepared and analyzed over a period of several days, so that day-to-day variations are reflected in the precision data; however, this is not a requirement.

11.2.2. Calculate the mean recovery and standard deviation for the analyte. Use the following equation to calculate the **LOD**:

LOD = SD of n samples * t

SD = standard deviation
n = sample size (7-10)
t = student's t value for the 99% confidence level with n-1 degrees of freedom where n is the number of replicates. (single-tailed) (**Table 9**)

Table 9. Student's t value for calculating LOD

n	t
7	3.143
8	2.998
9	2.896
10	2.821

11.2.3. The **LOD** for IBA and Azadirachtin is then used to calculate a **LLOQ** by multiplying the **LOD** by a factor a factor of 5 is required.

11.2.4. The **LLOQ** for IBA and and Azadirachtin must then be verified by extracting and analyzing a **MS** that has been spiked at no more than twice the **LLOQ**. The measured value must be within 80-120% of the expected value, and the detected peak must have a signal-to-noise ratio of >3.

11.2.5. Annually, or when minor changes to the method are made, an **LOD** verification must be completed as described in **Section 11.2.4** to demonstrate continued sensitivity at the **LLOQ**.

11.2.6. A new **LOD** study must be verified annually on each instrument, for each analyte, in each matrix. **LOD**'s are also determined each time that there is a significant change in the test method or instrument type.

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 **LLOQ** 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 **LLOQ** 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 twenty samples or one per sample set (all samples that were extracted within a 24-h period), whichever is more frequent. The **LFB** is fortified with the target analyte at a mid-level concentration.

11.5.2. The accuracy is calculated as percent recovery. The recovery for the analyte must be 70 – 130% of the true value.

11.6. Matrix Blank (**MB**)

11.6.1. The laboratory must analyze at least one matrix blank (**MB**) with every twenty samples or one per sample set (all samples extracted within a 24-h period), whichever is more frequent. 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, consult your supervisor on how to move forward.

11.6.2. Background contamination found must be <1/3 the **LLOQ** 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 every twenty samples or one per sample set, whichever is more frequent. The **MS** is fortified with the target analyte at a mid-level (100 ng/mL) concentration.

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

11.7.3. 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.

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

12.0. Procedure

12.1. Sample Preparation:

12.1.1. Weigh 500 ± 25 mg of the material directly into a tared 2-mL centrifuge tube.

12.1.2. Spike 400 ng (10 µL, 40 µg/mL) **IWD** (**Section 8.5**).

12.1.3. Add up to 1000 µL extraction solvent (**Section 8.2**)

12.1.4. Vortex for 30 seconds.

12.1.5. Place on the shaker for 15 minutes.

12.1.6. Centrifuge for 5 minutes at 12,000 RPM (13,362 × g).

12.1.7. Transfer approximately 600 µL of the supernatant to an amber vial for storage.

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

12.2.1. Weigh 500 ± 25 mg matrix (**Section 8.12**) directly into a tared 2-mL centrifuge tube.

12.2.2. Spike 100 ng (10.0 µL, 10000 ng/mL) Primary working solution of IBA (**Section 8.7**).

12.2.3. Spike 400 ng (10 µL, 40 µg/mL) **IWD** (**Section 8.5**).

12.2.4. Add up to 1000 µL extraction solvent (**Section 8.2**).

12.2.5. Vortex for 30 seconds.

12.2.6. Shake for 15 minutes.

12.2.7. Centrifuge for 5 minutes at 12,000 RPM (13,362 × g).

12.2.8. Transfer approximately 600 µL of the supernatant to an amber vial for storage.

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 12.1.7**.

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 12.2.8.**

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 **LLOQ**, **CCV**, and **CCR-WS**, followed by a **LRB**.

12.6.5.2. The **LLOQ**, **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**, **LLOQ**, and **LRB** at the end of the batch.

12.7. LC-MS/MS Integration/Quantitation Parameters

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

12.7.2. When the software inadequately integrates peaks, manual integration is necessary. See LOAC-011-SOP for guidance when integrating peaks manually.

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)	30.0	30.0	30.0
Expected RT (min)	8.63	9.60	9.63
Smoothing Width	3	3	3
Use Relative RT	No	No	No
Automatic – IQAIII	No	No	No
Specify Parameters-MQ III	Yes	Yes	Yes
Noise Percent	50	50	50
Peak Splitting Factor	0	0	0
Base Sub Window (min)	0.2	0.2	0.2
Report Largest Peak	No	No	No

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 identification is considered positive.

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 quant/qual ratios are listed in Table 10.

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

Analyte	Quantifying Transition	Qualifying Transition	Average Ratio	± 3 Standard Deviation	Ratio Range

	(Da)	(Da)			
IBA	202 / 116	202 / 158	1.026	0.508	0.518 – 1.534
Azadirachtin	719 / 659	719 / 687	2.092	0.336	1.756 – 2.428

13.2.3. The width of the relative retention time window used to make identifications is based upon measurements of actual retention time variations of standards 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 will weigh heavily in the interpretation of the chromatograms.

13.2.4. Current retention time windows are set to allow less than 2 % deviation from the expected retention times for all analytes.

13.2.5. 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.

13.3.2. Initial calibrations of the IS

13.3.2.1. Use the instrumental software and specified parameters to perform peak integration for all identified peaks.

13.3.2.2. The IS is used for response and retention time reference.

13.3.2.3. Calculate the average retention time of the IS in the calibration curve. The retention time of each standard IS must be within 2% of the average retention time of the standards in the curve.

13.3.2.4. Calculate the average response of the IS in the calibration curve. The response must be ≤ 10% for each of the standards in the calibration curve.

13.3.2.5. Calculate the retention time of the IS in each sample. The retention time of the sample must be within 2% of the average retention time of the standards in the curve.

13.3.2.6. Calculate the average **IS** peak area for each matrix (solvent, olive oil). Evaluate the system stability by using the following equation on every injection, and comparing to the criteria set in **Section 10.3**.

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

Where: IS_I = IS peak area for individual injection
IS_A = IS peak area average for given matrix

13.3.2.7. 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**.

13.3.3. QC and unknown samples

13.3.3.1. 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.

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

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

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.

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

13.3.4.1. 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.

13.3.5. 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**, unless the low-level standard calibration was removed, in which case the **LLOQ** must be adjusted accordingly.

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

13.4.3. Analyte(s) detected at a concentration at or above the **LOD**, but below the **LLOQ** are reported as less than (<) the **LLOQ** 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. Use the approximate concentration to adjust the sample size. If there is not enough for a new preparation, report as greater than (>) the **ULOQ**.

13.4.5.1. For example, if a concentration of 1250 ng/mL (2500 ppb) is measured, prepare a new sample using 250 mg of sample instead of 500 mg and follow Section 12.1.2 – 12.1.7 as before. The newly prepared sample should be about 625 ng/mL.

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 quality control 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 10.3.4**), calibration curve response (**Section 10.3.5**), and internal standards response (**Section 13.3.1**).

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 for routine sample batches.

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 **LLOQ** of 70% to 130% recovery for routine sample batches.

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 **LLOQ** was achieved, all affected samples must be re-analyzed after an acceptable **LLOQ** is achieved.

14.7. Failure to meet required QC criteria for System Blank of <1/3 **LLOQ** for target analyte(s) in routine sample batches.

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 manufacture'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 **LLOQ**, 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 **LLOQ** 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 **LLOQ**, 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 LLOQ 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 LLOQ, 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 13.3.2.6**) 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. In addition, notify immediate supervisor of the issue.

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 10.3**.

14.13.1. Check to determine if there is a sample preparation error.

14.13.2. Check if there is an interference peak co-eluting with IS.

14.13.3. If the **IS** variation is higher than 20%, a problem investigation must be performed until the reason for this variation is determined and the issue is resolved. The samples are then re-analyzed and reported. 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.

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 Quality Assurance Officer (QAO) for the Medical Marijuana Lab (MML).

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 are disposed of in red, 5-Gallon solvent cans.

16.4. Dispose of water 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.

16.6. Contact the Wadsworth Center Safety Office (473-8034) – ESP B940, for additional information or for information on the disposal of products not described in this SOP

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).

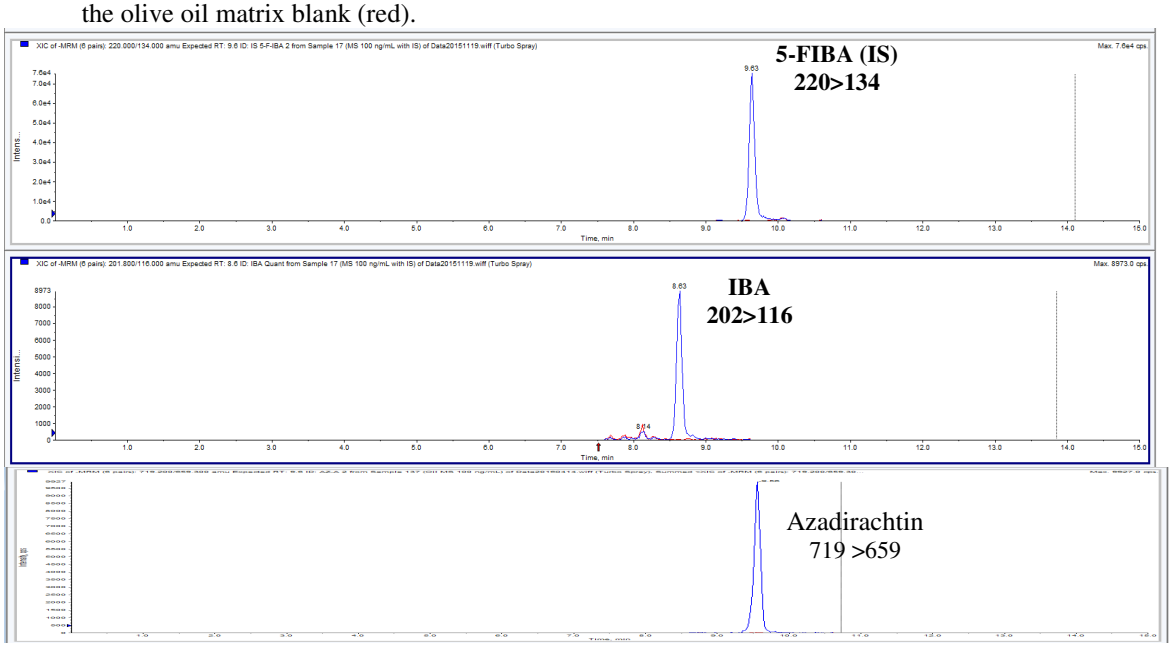
18.0. Supporting Documents

18.1. Appendix A – Tables and figures

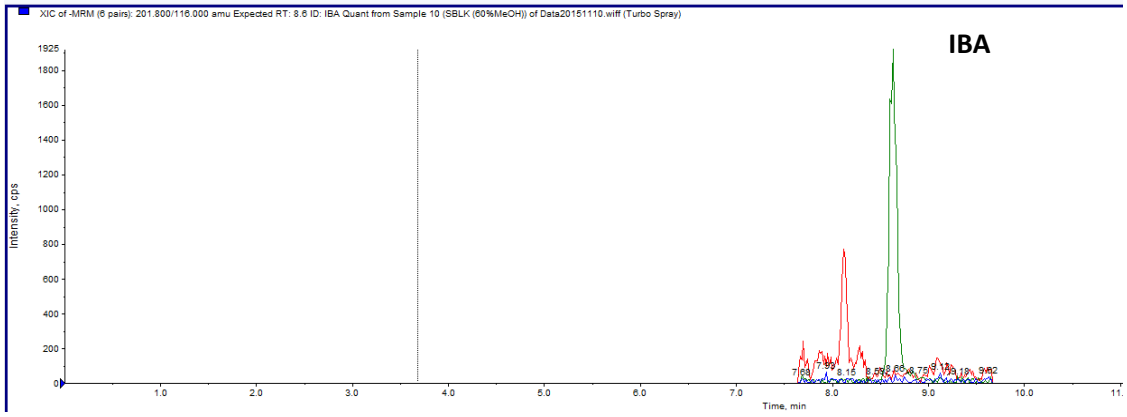
APPENDIX A

1.0. Example Chromatography

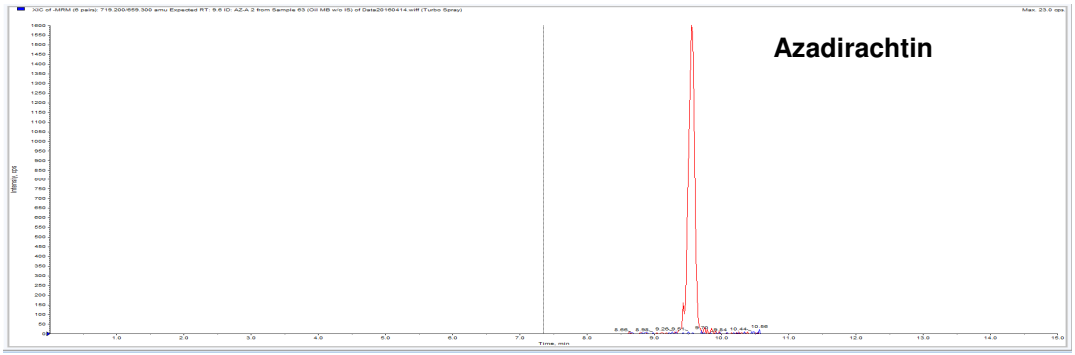
1.1. **Figure 1.** Chromatogram of the medium level of spiked extraction (100 ng/mL IBA and Azadirachtin spike) in olive oil matrix. The 100 ng/mL, equivalent to 200 ppb IBA and Azadirachtin spike in olive oil matrix (blue), is shown overlaid on the chromatogram recorded for the olive oil matrix blank (red).



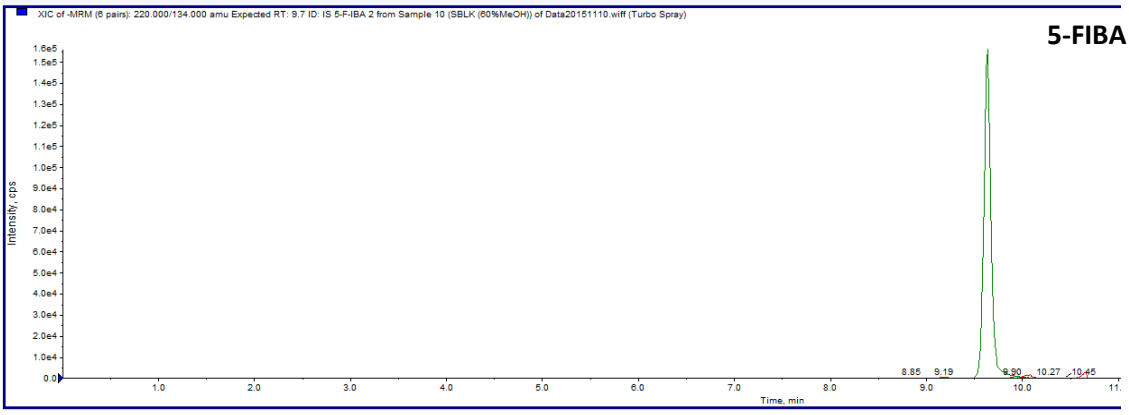
1.2. CalS-2b, 10 ng/mL IBA (green) overlaid with system blank (blue), and olive oil matrix blank (red).



1.3. CalS-2b at 10 ng/mL Azadirachtin (red), overlaid with those from the analysis of the system blank (grey), the olive oil matrix blank (blue) and tincture matrix blank (green).



1.4. CalS-2b, 400 ng/mL 5-FIBA (internal standard, green) overlaid with system blank (blue), and olive oil method blank (red).



2.0. Example Analysis Parameters

2.1. MS/MS Non-specific parameters:

Parameter	Value
MS Acquisition Time:	7 – 11.0 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 °C
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

2.2. MS/MS Analyte-specific parameters.

Quantifying product ions and CE are bold, qualifying product ions and CE are in (parenthesis).

Analytes	Polarity	Precursor ion	Product ion	Collision energy (eV)	Scheduled MRM analysis window (min)
IBA	Negative	202	**116** (158)	**-25** (-17)	7.62 – 9.62
Azadirachtin	Negative	719	**659** (687)	**(-20)** (-15)	9.50 – 9.70
5-FIBA, (IS)	Negative	220	**134** (147)	**-50** (-18)	8.65 – 10.65

2.3. HPLC parameters

Column	Value
Column:	Agilent Poroshell 120 EC-C18, 2.7 µm, 3.0 x 150 mm, # 693975-302
Guard Column:	Phenomenex SecurityGuard, Analytical Guard Cartridge System; # KJ0-4282
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)
6.0 min:	Divert to MS detector (Position B)
10.0 min:	Divert to waste (Position A)
HPLC Gradient, Time	**Percentage of Mobile Phase B**
Initial:	10%
2.0 min:	50%
10.0 min:	58%
11.0 min:	95%
15.0 min:	95%
15.1 min:	10%
20.0 min:	10% (End of Run)

3.0. Example Integration / Quantitation Parameters

3.1. 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)	30.0	30.0	30.0
Expected RT (min)	8.63	9.60	9.63
Smoothing Width	3	3	3
Use Relative RT	No	No	No
Automatic – IQAIII	No	No	No
Specify Parameters-MQ III	Yes	Yes	Yes
Noise Percent	50	50	50
Peak Splitting Factor	0	0	0
Base Sub Window (min)	0.2	0.2	0.2
Report Largest Peak	No	No	No