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Laboratory of Organic and Analytical Chemistry–
NYS ELAP Laboratory ID 10763

Division of Environmental Health Sciences
Albany, New York

Determination of the Pesticide Myclobutanil and the Synergist Piperonyl Butoxide in Medical Marijuana using LC-MS/MS with positive ion detection
NYS DOH MML-307

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1.0. Scope and Application

1.1. This method (NYS ELAP Method ID 9949) is to be used in the analysis of medical marijuana (MM) products for the quantitative determination of residual concentrations of pesticide Myclobutanil and Piperonyl butoxide (**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 Myclobutanil and/or Piperonyl butoxide is divulged by the Registered Organizations (ROs).

Table 1. Analyte List

Analyte	CAS Number	LOD[1] (ng/mL) in Solvent	LOQ[2] (ng/mL) in Solvent	LOD[1] (ng/g) in MCT[3] Matrix	LOQ[2] (ng/g) in MCT[3] Matrix
Myclobutanil	88671-89-0	0.7	3.5	14	70
Myclobutanil-(phenyl-d_4) (Internal Standard)	N/A	N/A	N/A	N/A	N/A
Piperonyl butoxide	51-03-6	0.23	2.5	5.0	50
Piperonyl butoxide-d_9 (Internal Standard)	N/A	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 (LOQ) – The minimum concentration that can be quantitatively reported for a target analyte (Section 3.16).
[3] MCT Matrix is a Medium Chain Triglyceride. See Table 3.

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

After sample extraction in methanol (MeOH), the samples are analyzed using an LC-MS/MS system operating in the positive ion electrospray ionization (ESI) mode with multiple reaction monitoring (MRM) for specific detection of the analytes, myclobutanil and piperonyl butoxide, and their corresponding internal standards, myclobutanil-(phenyl-d_4) and piperonyl butoxide-d_9. Chromatography of these compounds is performed on a C_{18} reversed-phase column using a programed gradient of increasing organic modifier over a period of 15 minutes. The limit of detection (LOD) and limit of quantitation (LOQ) for myclobutanil in matrix were 14 ng/g and 70 ng/g, respectively. The LOD and LOQ for piperonyl butoxide in matrix were 5.0 ng/g and 50 ng/g, respectively.

3.0. Definitions

3.1. Internal Standard (**IS**)– A pure compound that is not found in any sample. The IS compound is 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. Synonym: Method Blank.

3.6. Laboratory Fortified Blank (**LFB**) – An aliquot of extraction solvent that is spiked with known quantities of target analytes 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 and Laboratory Control Sample.

3.7. Matrix Blank (**MB**) – An aliquot of a "representative" 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. An **MS** is used, for example, to determine the effect of the matrix on the recovery efficiency of a method. When a sample is not suitable, a "representative" matrix may be used in its place. Synonym: Laboratory Fortified Sample Matrix.

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: Dilution Standard Solution.

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. Synonym: Continuing Calibration Check Standard.

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. Synonym: Secondary Source

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.

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 highest **CalS** standard.

3.18. Demonstration of Capability (**DOC**) - a procedure to establish the ability of the analyst to generate data with acceptable accuracy, precision, sensitivity and specificity using the method.

3.19. 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.

3.20. 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 myclobutanil, myclobutanil-(phenyl-d_4), piperonyl butoxide and piperonyl butoxide-d_9. According to the Safety Data Sheets (SDSs), myclobutanil and piperonyl butoxide are toxic. Those working with these analytes must always wear gloves, lab coats and safety glasses, and operate in a well-ventilated hood when working with these materials. Immediate hand washing 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 Safety Data Sheets (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 an **LRB** as described in **Section 11.4.**

5.1.1. Glassware and syringes used in the medical marijuana lab must be thoroughly cleaned 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 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 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 Laboratory 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**.

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 can occur because of contaminants present in the sample. If matrix interference is believed to have occurred, an **MS** should 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. Equipment and Supplies

Catalog numbers are included for illustration only and refer to products currently used in the laboratory. This is not a fully inclusive list and should not imply endorsement of any specific product(s). Instrumentation, equipment and supply substitutions are acceptable as long as substitutions meet the method criteria

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 amber autosampler vial with 0.3-mL target poly-spring inserts and Teflon-lined screw-caps or crimp-top caps.

6.1.6. Micropipette controller, various sizes, Eppendorf, Research Plus, or equivalent.

6.2. Sample Preparation Equipment

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

6.2.2. 2-mL amber autosampler vial with 0.3-mL target polyspring inserts and Teflon-lined screw-caps or crimp-top caps.

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

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

6.3. Instrumentation

6.3.1. An LC-MS/MS system with all the required accessories including: syringes, analytical columns, mobile phases, detectors, and a data system (*e.g.*, a Shimadzu HPLC interfaced with an AB Sciex 4500 QTRAP, or equivalent). The mass spectrometer system must be

capable of running multiple reaction monitoring (MRM) methods, or an equivalent type of method.

6.3.1.1. The Shimadzu HPLC system used to develop the method includes:

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

6.3.1.1.2. Pumps; model # LC-20ADxR.

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

6.3.1.1.4. Autosampler; model # SIL-20ACxR.

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

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

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.

6.3.1.2. The tandem mass spectrometer system used to develop the method includes:

6.3.1.2.1. AB Sciex 4500 Mass Spectrometer; model # API-4500 QTRAP.

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.

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.

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. Miglyol – (Miglyol 812, Warner Graham Co., Catalog # 140325, or equivalent).

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

7.1.6. 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. When available, the standards must be NIST traceable and are preferably ISO Guide 31 and 35 traceable, when possible.

7.3. The commercial standards listed below 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
Myclobutanil (Primary Source)	ULTRA Scientific, Inc.	PST-2470A100A01	100 µg/mL	Acetonitrile
Myclobutanil (Secondary Source)	AccuStandard	P-330S-10X	1000 µg/mL	Methanol
Myclobutanil-(phenyl-d_4) (IS)	Sigma-Aldrich	08977-5MG	N/A	Solid
Piperonyl butoxide (Primary Source)	ULTRA Scientific, Inc	PST-820I100A01	100 µg/mL	Isooctane (2,2,4-trimethyl pentane)
Piperonyl butoxide (Secondary Source)	Chem Service, Inc.	N-13061-100MG	N/A	Solid
Piperonyl butoxide (Secondary Source)	Chem Service, Inc.	S-13061A1-1ML	100 µg/mL	Acetonitrile
Piperonyl butoxide-d_9 (IS)	Toronto Research Chem, Inc.	P490202	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 accompanying NIST-traceable documentation. The preparation method, date of preparation, expiration date, and analyst must be traceable in laboratory documentation.

8.1.2. Standards (**Table 2)** and standard preparations noted in the following sections are examples of stocks and solutions currently used in the laboratory. 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 amber autosampler vial 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. **IS – Myclobutanil-(phenyl-d_4)** @100 µg/mL**.** Store at ≤ -20 °C for up to 12 months in a sealed vial or ampule.

8.2.1. Take an accurately weighed 1 mg (±0.02 mg) reference standard of myclobutanil-(phenyl-d_4) in a calibrated 10-mL volumetric flask (certified "A" class).

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

8.2.3. The **IS** solution is filled and sealed in a 2-mL crimp-cap amber autosampler vial and labeled.

8.3. **IS** – **Piperonyl butoxide-d_9** @1000 µg/mL. Store at ≤ -20 °C for up to 12 months in a sealed vial or ampule.

8.3.1. Take an accurately weighed 1 mg (±0.02 mg) reference standard of piperonyl butoxide-d_9 in a calibrated 1-mL volumetric flask (certified "A" class).

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

8.3.3. The **IS** solution is filled and sealed in a 2-mL crimp-cap amber autosampler vial and labeled.

8.4. Internal Standard Stock Diluent **(ISD) - myclobutanil-(phenyl-d_4)** and **piperonyl butoxide-d_9** @ 10 µg/mL. Store at ≤ -20 °C for up to 12 months in a sealed vial or ampule.

8.4.1. Fill a 1-mL volumetric flask 3/4 full with methanol.

8.4.2. Add 100 µL **IS myclobutanil-(phenyl-d_4)** @100 µg/mL **(Section 8.2)**.

8.4.3. Add 10 µL **IS piperonyl butoxide-d_9** @ 1000 µg/mL **(Section 8.3)**.

8.4.4. Dilute to volume with methanol and invert 3x to mix well.

Equation 1. Final concentration calculated as:

IS myclobutanil-(phenyl-d_4) @ 10 µg/mL

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

X= 10 µg/mL

Equation 2. Final concentration calculated as:

ISD piperonyl butoxide-d_9 @ 10 µg/mL

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

X= 10 µg/mL

8.4.5. One mL of the solution is transferred and sealed in a 2-mL amber autosampler vial and labeled.

8.5. Internal Standard Working Diluent **(IWD) myclobutanil-(phenyl-d_4) @ 20 ng/mL** and **piperonyl butoxide-d_9 @ 20 ng/mL.** Store at ≤ -20 °C for up to 12 months in a sealed vial or ampule.

8.5.1. Fill a 5-mL volumetric flask 1/2 full with methanol.

8.5.2. Add 10 µL of **IS myclobutanil-(phenyl-d_4)** @ 10 µg/mL and of **ISD piperonyl butoxide-d_9** @ 10 µg/mL **(Section 8.4).**

8.5.3. Dilute to volume with methanol and invert 3x to mix.

Equation 3. Final concentrations of **(IWD) myclobutanil-(phenyl-d_4)** and **piperonyl butoxide-d_9** calculated as:

IWD myclobutanil-(phenyl-d_4) and **piperonyl butoxide-$d9$ @** 20 ng/mL

(10 µg/mL) (10 µL) = (X µg/mL) (5000 µL)

X= 0.02 µg/mL or 20 ng/mL

8.5.4. The solution is transferred and sealed in 2-mL amber autosampler vial and labeled.

8.6. Primary Working Standards (**PS-WS**) – @1 µg/mL for myclobutanil and piperonyl butoxide. Store at ≤ -20 °C for up to 12 months in a sealed vial or ampule.

8.6.1. Fill a 1-mL volumetric flask 1/2 full with methanol.

8.6.2. Add 10 µL of myclobutanil (Primary Source) and piperonyl butoxide (Primary Source) both @ 100 µg/mL **(Section 7.3)**.

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

8.6.4. The P-**WS** solution is filled and sealed in a 2-mL crimp-cap amber autosampler vial and labeled.

8.7. Secondary Standard Stocks.

8.7.1. Piperonyl butoxide @ 100 µg/mL and Myclobutanil and 100 µg/mL. Store at ≤ -20 °C for up to 12 months in a sealed vial or ampule.

8.7.1.1. Take an accurately weighed 10 mg (±0.5 mg) reference standard of piperonyl butoxide **(Section 7.3)** in a calibrated 10-mL volumetric flask (certified "A" class).

8.7.1.2. Add 1000 µL of myclobutanil (Secondary Source) @ 1000 µg/mL (see Table 2).

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

8.7.2. The solution is filled and sealed in a 2-mL amber autosampler vial and labeled.

8.8. Secondary Working Standard (**CCR-WS**)– @ 1 µg/mL for myclobutanil and 1 µg/mL piperonyl butoxide. Store at ≤ -20 °C for up to 12 months in a sealed vial or ampule.

8.8.1. Fill a 1.0-mL volumetric flask 1/2 full with methanol.

8.8.2. Add 10 µL of myclobutanil (Secondary Source) @ 100 µg/mL and piperonyl butoxide (Secondary Source) @ 100 µg/mL **(Section 8.7).**

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

8.8.4. This solution is transferred and sealed in a 2-mL crimp-cap amber autosampler vial and labeled.

8.9. Mobile Phases

8.9.1. Mobile phase A: 15 mM ammonium formate (aq). The solution is stored at room temperature and must be prepared monthly

8.9.1.1. Fill a 1000 ±0.30 mL volumetric flask half full with water.

8.9.1.2. Add 945.9 ±15 mg of ammonium formate to the flask.

8.9.1.3. Dilute to volume with water and invert 3x to mix.

8.9.2. Mobile phase B: Methanol. The solution is stored at room temperature and must be changed at least every 12 months

8.10. Matrices

8.10.1. MCT is used as a "representative matrix" for the Demonstration of Capability **(DOC**), **LOD, MBLK, MS** and **MSD**.

Table 3. Matrices

Matrices	Manufacturer	Catalog #
Miglyol – (MCT) Medium Chain Triglyceride - Oil based matrix	Warner Graham Co	140325

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 the 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 medical marijuana products must be stored under the conditions recommended by the manufacturer. The storage is documented.

9.2.3. All MM products must be stored under the conditions recommended by the manufacturer(s) prior to analysis.

9.3. Sample Preservation and Storage:

9.3.1. Samples prepared in advance of instrumental analysis of a sample batch are stored in a freezer at ≤ -20 °C until analysis, which must be completed within 7 days of preparation. The samples are warmed to room temperature and vortexed to ensure homogeneity before analysis. Samples are placed in sealed containers and loaded into the autosampler tray, which is maintained at ≤ 4 °C during the analysis of the sample batch. If multiple sample batches are analyzed, the samples must be maintained at ≤ 4 °C in the autosampler for no longer than one week, and they must be kept away from light until analysis. After analysis is complete, the remaining sample is stored at ≤ -20 °C for one month, if reanalysis is necessary.

10.0. Calibration

10.1. LC-MS/MS

10.1.1. The *m/z* scale and resolution of the mass spectrometer must be calibrated 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 instrument manufacturer's instructions. The current optimal MS/MS conditions are described in **Tables 4** and **5**.

Table 4: General MS/MS parameters

Parameter	Value
MS Acquisition Time:	7.0 – 10.0 min
Curtain Gas Flow (CUR):	30.0 psi
Collision Gas Pressure (CAD Gas):	Medium (unitless)
Ion Transfer Voltage (IS):	4500.0 V
Temperature of Turbo Gas (TEM):	400.0 °C
Gas 1 – Nebulizer Gas (GS1):	45.0 psi
Gas 2 – Turbo Gas (GS2):	35.0 psi
Declustering Potential Voltage (DP):	80.0 V
Entrance Potential Voltage (EP):	10.0 V
Collision Cell Exit Potential (CXP):	4.0 V

Table 5: Analyte-specific MS/MS parameters

Quantifying product ions and CE are **bold**, qualifying product ions and their corresponding collision energies are in parenthesis.

Analytes	Polarity	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Scheduled MRM analysis window (min)
Myclobutanil	Positive	289.0	**70.0** (125.0)	**35** (25)	7.7 – 9.7
Myclobutanil-(phenyl-d_4)	Positive	293.0	**70.0** (129.0)	**35** (25)	7.7 – 9.7
Piperonyl butoxide	Positive	356.2	**177.2** (119.1)	**13** (47)	10.7–12.7
Piperonyl butoxide-*d*9	Positive	365.2	**177.2** (119.1)	**13** (47)	10.7–12.7

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

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

Table 6: HPLC parameters

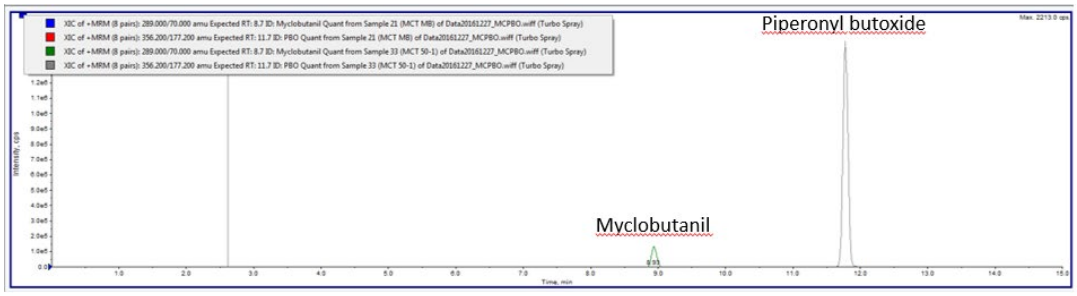
Column	**Value**
Column:	Agilent Poroshell 120 EC-C18, 2.7 µm, 3.0 x 150 mm, # 693975-302
Guard Column:	PhenomenexSecurityGuard, Analytical Guard Cartridge System; # KJ0-4282
Column Temperature:	40.0 °C
Autosampler	**Value**
Temperature:	4.0 °C
Injection Volume:	2.0 µL
Injection Loop Volume:	50.0 µL
Rinse Mode:	Before and after aspiration
Rinse Volume:	200 µL
Rinse Solvent:	Methanol
Mobile Phases	**Value**
Mobile Phase A:	15 mM ammonium formate
Mobile Phase B:	Methanol
Flow Rate:	0.400 mL/min
Initial Percentage MPB:	20%
Diverter Valve - Time	**Flow Destination (Position)**
Initial:	Divert to waste (Position A)
7.0 min:	Divert to MS detector (Position B)
10.0 min:	Divert to waste (Position A)

HPLC Gradient, Time	Percentage of Mobile Phase B
Initial:	20%
1.0 min:	50%
11.0 min:	100%
14.0 min:	100%
14.5 min:	20%
15.0 min:	20% (End of Run)

Table 7: Analyte and IS Retention Times

Analyte or IS	Expected Retention Time (min)	Retention Time Range (min)
Myclobutanil	8.90	7.90 – 9.90
Myclobutanil-(phenyl-d_4)	8.90	7.90 – 9.90
Piperonyl butoxide	11.70	10.70 –12.70
Piperonyl butoxide-$d9$	11.70	10.70 –12.70

Figure 1. Chromatogram of the medium level of spiked extraction (25 ng/mL myclobutanil and piperonyl butoxide) in oil matrix. The 25 ng/mL spiked sample, equivalent to 500 ng/g myclobutanil (green) and piperonyl butoxide (grey) spike in oil matrix, is shown overlaid on the chromatogram recorded for the oil matrix blank.



10.2. Calibration Curve Intermediate Preparation (**CalSI**)

Serial dilutions are made from the Primary Working Solution of myclobutanil and piperonyl butoxide (**Section 8.6**). The concentration range for myclobutanil and piperonyl butoxide is 2.5 ng/mL to 200 ng/mL. Store the calibration curve standards at ≤ -20 °C for up to 6 months in sealed vials or ampules.

10.2.1. **CalS6I** (200 ng/mL)

10.2.1.1. Dispense 200 µL Working Standard **(Section 8.6)** into 2-mL amber autosampler vial with a Teflon-lined screw cap, containing 800 µL of methanol.

10.2.1.2. Mix well by vortexing the autosampler vial at least 3 times for 10 s.

Equation 4. Final Concentration CalS6I calculated as:

$$(1000\ \text{ng/mL})\ (200\ \mu\text{L}) = (X\ \text{ng/mL})\ (1000\ \mu\text{L})$$

$$X = 200\ \text{ng/mL}$$

10.2.2. **CalS5I** (100 ng/mL)

10.2.2.1. Dispense 500 µL of CalS6I (**Section 10.2.1**) into a 2-mL amber autosampler vial with a Teflon-lined screw cap, labeled **CalS5I,** containing 500 µL of methanol.

10.2.2.2. Mix well by vortexing the amber autosampler vial at least 3 times for 10 s.

Equation 5. Final Concentration CalS5I calculated as:

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

X=100 ng/mL

10.2.3. **CalS4I** (50 ng/mL)

10.2.3.1. Dispense 500 µL of **CalS5**I (**Section 10.2.2**) into a 2-mL amber autosampler vial with a Teflon-lined screw cap, labeled **CalS4I,** containing 500 µL of methanol.

10.2.3.2. Mix well by vortexing the autosampler vial at least 3 times for 10 s.

Equation 6. Final Concentration CalS4I calculated as:

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

X= 50 ng/mL

10.2.4. **CalS3I** (10 ng/mL)

10.2.4.1. Dispense 200 µL of **CalS4I** (**Section 10.2.3**) into a 2-mL amber autosampler vial with a Teflon-lined screw cap, labeled **CalS3I,** containing 800 µL of methanol.

10.2.4.2. Mix well by vortexing the autosampler vial at least 3 times for 10 s.

Equation 7. Final Concentration CalS3I calculated as:

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

X=10 ng/mL

10.2.5. **CalS2I** (5 ng/mL)

10.2.5.1. Dispense 500 µL of **CalS3I** (**Section 10.2.4**) into a 2-mL amber autosampler vial with a Teflon-lined screw cap, labeled **CalS2I,** containing 500 µL of methanol.

10.2.5.2. Mix well by vortexing the autosampler vial at least 3 times for 10 s.

Equation 8. Final Concentration CalS2I calculated as:

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

X=5 ng/mL

10.2.6. **CalS1I** (2.5 ng/mL)

10.2.6.1. Dispense 500 µL of **CalS2I** (**Section 10.2.5**) into 2-mL amber autosampler vial with a Teflon-lined screw cap labeled **CalS1I** containing 500 µL of methanol.

10.2.6.2. Mix well by vortexing the autosampler vial at least 3 times for 10 s.

Equation 9. Final Concentration CalS1I calculated as:

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

X=2.5 ng/mL

10.2.7. Cross check reference standard working solution (CCR-WS) @ 50 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.

10.2.7.1. Dispense 10 µL of secondary working standard **(SWS) @** 1.0 µg/mL (**Section 8.8**) and 190 µL of MeOH into 2-mL amber autosampler vial with a Teflon-lined screw cap labeled **CCR-WS** containing 200 µL of **IWD** @ 20 ng/mL myclobutanil-(phenyl-d_4), 20 ng/mL piperonyl butoxide-*d*9 (see **Section 8.5**).

10.2.7.2. Mix well by vortexing the autosampler vial at least 3 times for 10 s

10.2.8. Prepare calibration standard mixtures with **IWD** by diluting 100 µL of each **CalSI** (**Section 10.2.1 – 10.2.6**) with 100 µL of **IWD** @ 20 ng/mL myclobutanil-(phenyl-d_4), 20 ng/mL piperonyl butoxide-*d*9 (see **Section 8.5**) and mix well. Be sure to rinse solution down the sides of the container. Failure to mix well may cause a failure of the linearity requirements. Store the calibration standards at ≤ -20 °C for up to 6 months in sealed vials or ampules. The final concentrations are shown in **Table 8**.

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

Final curve Cal STD w/IWD	Myclobutanil and piperonyl butoxide final concentration (CalS) in solvent (ng/mL)	Std (STDI without IWD)	Ref Section ID Myclobutanil and piperonyl butoxide Intermediate Std (STDI - without IWD)	Volume of myclobutanil and piperonyl butoxide (STDI - without IWD)	IWD Ref ID	Volume of IWD	Myclobutanil and piperonyl butoxide final concentration in matrices (ng/g)
CalS6	200	CalS6I	10.2.1	100 µL	8.5	100 µL	4000
CalS5	100	CalS5I	10.2.2	100 µL	8.5	100 µL	2000
CalS4	50	CalS4I	10.2.3	100 µL	8.5	100 µL	1000
CalS3	10	CalS3I	10.2.4	100 µL	8.5	100 µL	200
CalS2	5	CalS2I	10.2.5	100 µL	8.5	100 µL	100
CalS1	2.5	CalS1I	10.2.6	100 µL	8.5	100 µL	50

10.2.9. After at least one system blank injection, start with the lowest standard concentration (**CalS1**) and analyze each calibration standard. Tabulate the responses (analyte peak area/IS peak area ratios) and use them 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 myclobutanil and piperonyl butoxide 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 **LOQ** listed in **Section 1.1** for the analyte, or the **LOQ** must be adjusted accordingly.

10.3.3. The absolute **IS** response in each chromatographic run must not deviate by more than 20% 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, must be within 80-120% of the true value for each analyte for the initial calibration to be considered valid. The exception is at or below the **LOQ**, 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.

10.3.6. If these criteria cannot be met, a new calibration must be established.

10.4. Initial Verification of Calibration

10.4.1. The initial calibration for each analyte must be verified by analyzing a Cross Check Reference Standard Working Solution (**CCR-WS**) @ 50 ng/mL.

10.4.2. The measured recovery value of **CCR-WS** must be within 80 – 120% of the true value for each analyte.

10.5. Continuing Calibration Check

10.5.1. The calibration curve and average response factors must be verified on each working day. 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. 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.

The response for any analyte in the **LOQ** standard must be within ±30% of the predicted response. The **CCVs** must be within ±20% of the predicted response.

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

10.5.4. Examples of typical **CCV** concentrations analyzed include:

10.5.4.1.1. 5ng/mL, 10 ng/mL, and 50ng/mL (same as **CalS2** thru **5** – (**Section 10.2, Table 8**)

10.5.4.1.2. **LOQ** is prepared at 2.5 ng/mL(same as **CalS1**).

11.0. Quality Control/Assurance

11.1. Demonstration of Capability (DOC)

11.1.1. Initial DOC - Each analyst must perform an initial demonstration of capability using the procedures described in this SOP for the target analyte(s). The initial DOC must consist of the analysis of four or five **MS** samples that have been fortified with the 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. For each 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.

11.1.2. Continuing DOC - Annually, each analyst must complete a continuing DOC by one of the following methods.

11.1.2.1. Acceptable performance of a blind sample, such as an external proficiency test (PT) sample.

11.1.2.2. Internally prepared PT sample in which the concentrations of all analytes have been accurately measured.

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

11.1.3. 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**. Minor changes to the method are evaluated using the **MS** per **Section 11.7** or the CCR-WS per **Section 10.4**.

11.2. Limit of Detection (LOD) and Limit of Quantitation (LOQ)

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 methanol 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 of the **LOQ**.

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. An **LRB** 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). 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 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. 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 fortified spike concentration.

11.6. Method 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). 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 on how to proceed.

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

11.7. Matrix Spike and Matrix Spike Duplicates (**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 separately prepared MSD 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 fortified spike concentration.

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 Standard (IS)

11.8.1. See sections 10 and 13.

12.0. Procedure

12.1. Sample Preparation:

12.1.1. Option 1: LOQ as per method

12.1.1.1. Weigh 10 ±0.5 mg of the material directly into a tared 2-mL amber autosampler vial.

12.1.1.2. Add 190 µL methanol.

12.1.1.3. Retain at room temperature until the sample material is fully dissolved and vortex for 1 min.

12.1.1.4. Into a 2-mL vial with insert, add 30 µL of dissolved sample and 30 µL **IWD (Section 8.5).**

12.1.1.5. Vortex for 30 s.

12.1.1.6. Transfer the mixture to an amber autosampler vial for storage.

12.1.2. Option 2: Sample Preparation with dilution, increasing the LOQ

Recently, the MM program was provided a guideline of < 1.0 ppm PBO for the maximum contaminant level. The laboratory routinely dilutes samples, to extend column life and/or to keep samples within the calibration range. The LOQ is adjusted for this dilution. For example, the

LOQ in the sample is adjusted to account for the 5-fold dilution, and reported at < 250 ng/g PBO in the sample.

12.1.2.1. Weigh 10 ±0.5 mg of the material directly into a tared 2-mL amber autosampler vial.

12.1.2.2. Add 990 µL methanol.

12.1.2.3. Retain at room temperature until the sample material is fully dissolved and vortex for 1 min.

12.1.2.4. Into a 2-mL vial with insert, add 30 µL of dissolved sample and 30 µL IWD **(Section 8.5).**

12.1.2.5. Vortex for 30 s.

12.1.2.6. Transfer the mixture to an amber autosampler vial for storage.

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

12.2.1. Weigh 10 ±0.5 mg of the matrix **(Section 8.10)** into a tared 2-mL amber autosampler vial.

12.2.2. Spike 10.0 µL, CalS6 @ 200 ng/mL (**Section 10.2.1**) into the amber autosampler vial.

12.2.3. Add 980.0 µL methanol.

12.2.4. Retain at room temperature until fully dissolved and vortex for 1 min.

12.2.5. Into a separate 2-mL amber autosampler vial with insert, add 30 µL of dissolved sample and 30 µL **IWD (Section 8.5).**

12.2.6. Vortex for 30 seconds.

12.2.7. Transfer the mixture to an amber autosampler vial for storage.

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

12.3.1. Label a 2-mL amber autosampler vial with insert.

12.3.2. Add 30 µL of MeOH

12.3.3. Add 30 µL **IWD (Section 8.5)**.

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

12.4.1. Label a 2-mL amber autosampler vial.

12.4.2. Add 990 µL of methanol directly into a tared 2-mL amber autosampler vial.

12.4.3. Add 10.0 µL, WS @ 1000 ng/mL (**Section 8.6**) into the amber autosampler vial.

12.4.4. Follow the steps in **Section 12.2.4** through **Section 12.2.7.**

12.5. Matrix Blank (**MB**)

12.5.1. Label a 2-mL centrifuge amber autosampler vial.

12.5.2. Follow the steps in **Section 12.2**, omitting instruction **12.2.2** in which the standards are spiked. All other steps are identical.

12.6. LC-MS/MS Analytical Procedure.

12.6.1. Perform an LC-MS/MS calibration check (**Section 10.1**). These parameters are checked during routine preventive maintenance with the instrument manufacturer. The frequency is based on the instrument manufacturer's recommendations.

12.6.2. Equilibrate the LC-MS/MS system with mobile phase flowing at the initial parameters described in **Table 6** in **Section 10.1.3**.

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

12.6.4. Analyze a full initial calibration (**Section 10.2 and Section 10.3**), followed by an **LRB** to assess potential carry-over.

12.6.4.1. Analyze a **CCR-WS** followed by an **LRB**.

12.6.4.2. 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. After the initial calibration curve is established, subsequent batches can be verified as follows:
12.6.5.1. Analyze an **LOQ** and a **CCR-WS**, followed by an **LRB**.

12.6.5.2. The **LOQ**, **CCR-WS** and a **CCV** bracketing the analysis batch 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 a CCV every 10 samples.

12.6.8. Analyze a bracketing standard at the end of each batch of 20 samples.

12.7. LC-MS/MS Batch

12.7.1. **Table 10** depicts an example of a full 20-sample batch together with a full calibration curve.

Table 10: Batch submission example

Injection #	Sample	Comments
1	SBLK	While only 1 blank is needed to check the instrument for interference, multiple injections may be needed to reduce noise (See **Section 14.7.4**).
2	SBLK	
3	SBLK	
4	LRB	
5	CalS-1	This example uses a full calibration curve. If a full calibration curve is not needed (see **Section 12.6.5**), only the LOQ (CalS-1) and a CCV (CalS-2, 3 or 4) is needed.
6	CalS-2	
7	CalS-3	
8	CalS-4	
9	CalS-5	
10	CalS-6	
11	LRB	Blank to check for carry-over.
12	CCR	Calibration curve cross-check.
13	LRB	Blank to check for carry-over.
14	LFB	Sample 1
15	MB	Sample 2
16	MS	Sample 3
17	MSD	Sample 4
18	Unknown 1	Sample 5
19	Unknown 2	Sample 6
20	Unknown 3	Sample 7
21	Unknown 4	Sample 8
22	Unknown 5	Sample 9
33	Unknown x	Up to sample 20 *
34	LRB	Blank to check for carry-over.
35	CCV (CalS-2 – CalS-5)	Verifies calibration curve is still valid.
36	LRB	Blank to check for carry-over.

* For batches with 20 or more unknown samples, additional CCVs are dispersed through out the analytical run, one per batch of 20.

12.8. LC-MS/MS Integration/Quantitation Parameters

12.8.1. The integration of peaks is done by the software whenever possible. **Table 11** lists the ideal parameters for integrating peaks using Analyst 1.6.1 software.

12.8.2. When the software inadequately integrates peaks, manual integration is necessary. Laboratory specific procedures must be used a guidance for any manual integration of peaks.

Table 11: All analytes (Quantifying transitions only)

Parameters	**Myclobutanil (target analyte)**	**Myclobutanil-(phenyl-d_4) (IS)**	**Piperonyl butoxide (target analyte)**	**Piperonyl butoxide-d_9 (IS)**
Q1/Q3	*m/z* 289.0 → 70.0	*m/z* 293.0 → 70.0	*m/z* 356.2 → 177.2	*m/z* 365.2 → 117.2
Min. Peak Height	0	0	0	0
Min. Peak Width	0	0	0	0
RT window (sec)	30.0	30.0	30.0	30.0
Expected RT (min)	8.90	8.90	11.70	11.70
Smoothing Width	3	3	3	3
Use Relative RT	No	No	No	No
Automatic – IQAIII	No	No	No	No
Specify Parameters-MQ III	Yes	Yes	Yes	Yes
Noise Percent	50	50	50	50
Peak Splitting Factor	0	0	0	0
Base Sub Window (min)	0.2	0.2	0.2	0.2
Report Largest Peak	No	No	No	No

13.0. Data Acquisition, Reduction, Analysis, and Calculations

13.1. HPLC

13.1.1. **Table 6** (**Section 10.1**) 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**. The standards and prepared samples must be in methanol.

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. A range of ± 15% of the average ratio can be calculated and established as the suggested tolerance for identification of a compound.

13.2.2.2. Current quantifying / qualifying peak area ratios are listed in **Table 12**.

Table 12: Quantifying / Qualifying MS/MS transition peak area ratios in samples.

Analyte	Quantifying Transition (*m/z*)	Qualifying Transition (*m/z*)	Average Ratio	±15% of Average Ratio	Ratio Range
Myclobutanil	289.0→70.0	289→ 125	4.44	0.66	3.77-5.10
Piperonyl butoxide	356.2→177.2	356.2→119.1	2.33	0.35	1.98-2.68

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 Calibration of the **IS**

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

13.3.2.2. The **IS** is used for area ratio determination 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 not deviate more than 20% for the average value 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 in each matrix. Evaluate the system stability by using the following equation on every injection and comparing the result to the criteria set in **Section 10.3**.

Equation 11.

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

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 level. 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 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:

Equation 12.

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

Where:

C_S = Concentration of analyte in sample (ng/g)
C_E = Concentration of analyte in solvent (ng/mL) (from software)
V_F = Final volume of sample (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%.

Equation 13.

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

Where:

RPD is in percent (%).
MS = Matrix Spike concentration in ng/g.
MSD = Matrix Spike Duplicate concentration in ng/g.

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. Nondetected 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 nondetects 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 125 ng/mL is measured, prepare a new sample using 5 mg of sample instead of 10 mg and follow Section **12.1.1.2 – 12.1.1.6** as before. The newly prepared sample should be about 62.5 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 reinjection 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 reanalyzed.

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 reanalyzed 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 13.3.2**).

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 reanalyzed; 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 **CCV** of 80% to 120% recovery.

14.5.1. A new **CCV** is prepared and reanalyzed; 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, analyzed 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 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-1** sample is prepared and reanalyzed; it may be necessary to prepare this from new working or stock solutions.

14.6.2. If a newly prepared **CalS-1** 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-1** 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 reanalyzed 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 contaminants are removed from the column based on the 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 reanalyzed. If reanalysis of suspect samples is not possible due to lack of remaining 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 reanalysis and qualification is not necessary.

14.8. Failure to meet required QC criteria for LRB 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 reanalyzed (if system contamination is suspected) or reextracted and analyzed (if extraction contamination is suspected). If reanalysis or reextraction of suspect samples is not possible due to lack of remaining 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 reanalysis 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 were previously analyzed, then any samples containing target analytes must be reanalyzed (if system contamination is suspected) or reextracted 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 reanalysis 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 **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 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, indicating that the spiking solution is not the problem, the **LFB** must be re-prepared.

14.11. Failure to meet required QC accuracy (recovery) criteria for **MS** as described in **Section 11.7**.

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 reanalysis 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 reanalyzed **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 **MS** 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 the **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 the **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 the **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 10.3.3**.

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 reanalyzed and reported. If reanalysis or reextraction of suspect samples is not possible due to lack of remaining 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 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 regarding 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 container 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 4L (1-Gallon).

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

16.4. Dispose of nonhazardous 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. 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).

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