Method Validation Report for the Identification and Quantitative Threshold Testing of Tetrahydrocannabinol (THC) in Cannabis

1. Purpose/Scope

This report describes the validation for the identification and quantitative threshold testing of delta-9-tetrahydrocannabinol (THC) in Cannabis. The Florida state defined limit specifies that for a Cannabis plant to be considered hemp, the plant has a THC concentration below 0.3 percent based on the dry weight of the plant. This method is designed to determine if a plant contains at least 1% THC (which is more than three times the state defined threshold). No effort is made to ensure 100% extraction efficiency or to determine the actual percentage of total THC present in the plant.

The original method validation for this procedure was conducted by the Drug Enforcement Administration – "Qualitative Analysis of Cannabinoids by Gas Chromatography"[1]. This method will ultimately be used in conjunction with existing testing protocols that are designed to identify Cannabis, but not differentiate its form. The total amount of THC in the plant is defined as the combined THC and delta-9-tetrahydrocannabinolic acid (THCA) concentration. Since THCA is converted to THC in the GC injection port, the method does not differentiate between THC and THCA. Using a 50 mg plant sample, 1% THC equates to 0.5 mg of THC extracted from the sample. Samples are extracted in 5 mL of solvent (internal standard solution), resulting in a 0.1 mg/mL solution. The ratio of the THC to internal standard (IS) from the extract is then compared to a known 0.1 mg/mL THC with IS positive control to determine if the plant sample is over the decision point (over 1% THC).

This method was deemed to be valid and suitable for the intended use. The validation demonstrates that GC-MS 1 is suitable for the analysis of plant material and for assessing if the concentration of THC is below or above 1%. During the validation, it was discovered that GC-MS 4 was not sensitive enough to successfully pass the criteria for the precision (reproducibility) experiment (Step 3). Therefore, GC-MS 1 will be utilized for the method since this instrument successfully passed the criteria in all steps of the validation. The data obtained for all experiments was evaluated using both peak heights and peak areas to calculate the THC:IS ratio. Using peak areas to calculate the THC:IS ratio provided more consistent data particularly in the precision experiments so peak area ratios will be used for this method. In addition, all analysts in the Chemistry Unit were competency tested and successfully passed these competency tests using GC-MS 1. Some steps of the validation were also performed on GC-MS 4 and are included in this validation report. This report documents the results of the semi-quantitative validation.

2. Analytical Method

Reagent and Control Preparation

- Internal Standard Solution (IS) – Prepare 0.075 mg/mL internal standard solution by weighing 75 mg of testosterone and diluting to 1000 mL using methanol (scale quantities up or down as needed). Note: The same lot of IS solution must be used to prepare all samples and corresponding positive controls in the same analytical batch. Store at room temperature.
- Positive Control – Prepare a 0.1 mg/mL THC positive control each day when an analysis is performed. Using a calibrated 100-1000 µL pipette, transfer 900 µL of internal standard solution into an autosampler vial. Using a calibrated 10-100 µL pipette, add 100 µL of THC certified reference material to the vial. Store the THC certified reference material in the freezer in an amber glass vial.

Sample Preparation

Crumble with gloved hands or cut into fine pieces two samples from buds and leaves of plant material of approximately 50 mg (±1 mg). Accurately weigh the plant material using weighing paper on a calibrated analytical balance and then place into two 13 x 100 mm labeled test tubes (seeds, stems, stalks or roots are not included). Record the weights of the samples. Dissolve each sample in 5 mL of internal standard solution, invert once and sonicate for at least 10 minutes. For each extraction, an extraction blank is also included. Invert the test tubes to mix the solution and decant approximately 1 mL of the IS solution into an autosampler vial for GC-MS analysis.

NOTE: This sample preparation procedure is not intended to produce complete decarboxylation of THCA or complete extraction of THC/THCA. Therefore, THC/IS ratios observed will be lower than values expected under full decarboxylation or dissolution conditions.

3. Reference Materials

- MS Reference Material – PFTBA is used to calibrate the MS in accordance with the procedures recommended by the instrument manufacturer.
- Internal Standard Reference Material -Testosterone, Lot #11H0756, purchased from Sigma, Saint Louis, MO.

Drug Reference Material	Lot Number
∆8-Tetrahydrocannabinol (∆8-THC)	0508237-9
∆9-Tetrahydrocannabinol (∆9-THC)	0567300
Cannabichromene (CBC)	0559373-6
Cannabigirol (CBG)	0500095-4
Phytocannabinoid Mix 3 [∆9-Tetrahydrocannabinol (∆9-THC), Cannabidiol (CBD), Cannabinol (CBN)]	0538899

Instrumental parameters

An Agilent 7890 series gas chromatograph (GC), coupled with an Agilent 5977 series mass spectrometer (MS), designated as GC-MS 1, will be used to perform this method. Part of the validation was also performed on an Agilent 6890 series gas chromatograph (GC), coupled with an Agilent 5973 series mass spectrometer (MS), designated as GC-MS 4. The configuration for these instruments are as follows:

Instrument Name	**GC Type**	**MS Type**	**Column**
GC-MS 1	Agilent 7890 series	Agilent 5977 series	Agilent HP-5MS UI Part #19091S-433UI; 30m x 0.250 mm i.d. x 0.25 µm film thickness [(5%-phenyl)-methylpolysiloxane]
GC-MS 4	Agilent 6890 series	Agilent 5973 series	Restek Rxi®-5ms Part #13420; 15m x 0.25 mm i.d. x 0.25 µm film thickness [Crossbond® 5% diphenyl / 95% dimethyl polysiloxane]

GC-MS Method (THCSCREN)		
	GC-MS 1	GC-MS 4
Inlet Temperature:	300^{o}C	300^{o}C
Injection Volume:	1 µL	1 µL
Injection Mode:	Split	Split
Split Ratio:	50:1	25:1
Injection Solvent:	Methanol with 0.075 mg/mL testosterone (internal standard)	Methanol with 0.075 mg/mL testosterone (internal standard)
Carrier Gas:	Helium, 2 mL/min, constant flow	Helium, 1.5 mL/min, constant flow
Temperature Program:	255^{o}C (3.5 min hold), ramp to 280^{o}C at 30^{o}C/min (0.9 min hold)	225^{o}C (3 min hold), ramp to 280^{o}C at 30^{o}C/min (0.5 min hold)
Total Run Time:	5.23 min.	5.33 min.
Mass Analyzer:	Single quadrupole	Single quadrupole
Ionization Mode:	Electron ionization (70 eV)	Electron ionization (70 eV)
Transfer Line Temperature:	280^{o}C	280^{o}C
Source Temperature:	230^{o}C	230^{o}C
Quad Temperature:	150^{o}C	150^{o}C
Solvent Delay:	0.93 min.	0.50 min.
Scan Range:	40-480 amu	40-480 amu
Scan Speed:	1,562 amu/s [N=2]	3.28 scans/s
Tune Type:	Standard	Standard

4. Performance Characteristics/Acceptance Criteria

4.1. Selectivity/Specificity (performed on GC-MS 1 and 4)	1. No interferences at the retention time for THC or the internal standard in five different extracted marijuana samples and one extracted hemp sample. 2. No interference at the retention time for THC from the internal standard. 3. No interference at the retention time for the internal standard from a high concentration of THC. 4. No interference at the retention time for THC and the internal standard from at least five similar cannabinoids (CBC, CBD, ∆8-THC, CBG, CBN) that are commonly identified in Cannabis samples (resolution > 1).	1. There was no interference at the retention time for THC or the internal standard in five extracted marijuana samples and one extracted hemp sample. 2. There was no interference at the retention time for THC from the internal standard in a 0.1 mg/mL positive control. 3. There was no interference from a high concentration of THC with the internal standard using a 4 mg/mL THC standard. 4. There was no interference at the retention time for THC or the IS from five similar cannabinoids that are present in Cannabis samples each at a concentration of 0.1 mg/mL. THC/delta-8-THC resolution (GC-MS 1 = 1.51, GC-MS 4 = 1.37); IS/CBN resolution (GC-MS 1 = 6.73, GC-MS 4 = 4.22)
4.2. Precision at the Decision Point (performed on GC-MS 1)	1. Precision at the decision point will be investigated by analyzing a standard mixture of THC in triplicate on five separate days. 2. Evaluated using THC standards at a concentration of no more than 50% below the decision point, at the decision point (0.1 mg/mL) and no more than 50% above the decision point. 3. The coefficient of variation (CV) of the THC:IS ratios for each concentration should be within 20%. 4. The mean of the decision point and the mean +/- 2SD of the above and below concentrations should not overlap.	1. Standards prepared at three different THC concentrations (0.05 mg/mL, 0.1 mg/mL and 0.15 mg/mL) were evaluated. 2. Standards were prepared on each day and run in triplicate on five separate. 3. The %CVs were 17.0, 13.5 and 8.6%, for the 0.05, 0.1 and 0.15 mg/mL concentrations, respectively. 4. The mean of the decision point (2.005) and the mean +/- 2SD of the 0.05 mg/mL (0.787 +/- 0.268) and the 0.15 mg/mL (3.293 +/- 0.566) did not overlap. 5. The relative retention time of THC at 0.10 mg/mL was within ±0.04 minutes with a %CV of 0.03% over the five day period.

4.3. Precision (Reproducibility) (performed on GC-MS 1 and 4)	1. Reproducibility will be investigated by analyzing one marijuana sample and one Hemp sample ten times. 2. The coefficient of variation (%CV) of the THC:IS ratios should be within 20%.	1. One marijuana case sample and one hemp sample were sampled ten times to evaluate the reproducibility of the method. 2. The %CV of the THC:IS area ratios for the marijuana and hemp were: GC-MS 1 – 7.0% and 13.2% GC-MS 4 – 6.8% and 25.5%
4.4. Trueness (performed on GC-MS 1)	1. Trueness will be investigated by analyzing a Cannabis sample (in duplicate) of a known concentration near the decision point. 2. The %CV of the duplicate samples should be within 20% of each other and either above or below the decision point based on the actual THC concentration of the Cannabis sample.	1. Five Cannabis samples of known concentration (one illicit Cannabis sample, three combination Cannabis/hemp samples and one hemp sample) were analyzed in duplicate and the concentration of THC was determined using this method. 2. The %CV of the duplicate samples were 3.2%, 7.3%, 4.3%, 2.2 and 14.1%, respectively. All samples were either above or below the decision point based on the quantitated THC concentration of the Cannabis sample.
4.5. Limit of Detection (LOD) (performed on GC-MS 1 and 4)	1. The LOD is defined as the value of the administratively-defined decision point concentration (0.1 mg/mL). 2. Signal-to-noise of 10:1 and good chromatographic peak shape.	1. Both THC and the IS had good chromatographic peak shape. 2. Signal-to-noise ratios for THC at 0.1 mg/mL on both GC-MS 1 and GC-MS 4 were greater than 10:1 (S/N = 202 and 615, respectively).
4.6. Ruggedness/robustness (performed on GC-MS 1)	1. Validation studies will be performed over multiple days and all analysts will be competency tested.	1. Validation studies were performed over multiple days and demonstrated repeatable results. All analysts were competency tested with 2 samples; one sample above the decision point and one sample below the decision point. %CV for duplicate samples were all below 20%.

4.7. Carryover (performed on GC-MS 1 and 4)	1. A blank sample must be analyte free when run after a standard prepared at or above a high concentration of THC. 2. Lack of carryover must be determined by triplicate analyses (repeated injection of a standard and blank is acceptable).	1. A blank IS sample was THC free when run after a standard prepared at a concentration of 4 mg/mL. 2. The lack of carryover was determined by triplicate analyses of a standard and IS blank sample.
4.8. Extract Stability (performed on GC-MS 1)	1. At least three replicates of marijuana and one Hemp sample will be extracted. The marijuana extracts will be combined and then divided into five different vials. A vial of each of the combined marijuana and the hemp sample will be injected in triplicate on day 0. The other vials will be stored on the instrument and reinjected on each subsequent day for three days in triplicate. 2. The THC response must be within ± 20% of the response from day 0. If the response falls outside this range then the extract stability of the analyte has been exceeded.	1. Three different marijuana samples and one hemp sample was extracted. The marijuana extracts were combined and then divided into five different vials. A vial of the marijuana extract and a vial of the hemp extract was injected in triplicate on day 0. The other vials were stored on the instrument and re-injected on days 1, 2, 3 and 4 in triplicate. 2. Extract stability was confirmed to be at least 4 days for THC after the day of extraction.

5. Validation Steps

Step 1: Selectivity/Specificity

Analyst: Amber Kohl (AK)
9/13/19 & 9/16/19

General Outline of Validation Step:
Part 1 (9/13/19)

- Accurately weigh five marijuana samples (50 mg (±1 mg) each) by crumbling with gloved hands onto weighing paper and put into labeled test tubes (seeds, stems, stalks or roots are not included).
- Accurately weigh one hemp sample (50 mg (±1 mg) each) by crumbling with gloved hands onto weighing paper and put into labeled test tubes (seeds, stems, stalks or roots are not included).

- Dissolve all samples in 5 mL of methanol containing internal standard solution (ISTD215), invert once to mix and sonicate for 10 minutes.
- One extraction blank is also included.
- Invert the test tubes to mix, transfer approximately 1 mL of the solution into an autosampler vial and analyze on both GC-MS 1 and 4.

Part 2 (9/13/19)

- Prepare a 0.1 mg/mL THC positive control (CON774) and analyze on both GC-MS 1 and 4.

Part 3 (9/16/19)

- Prepare a 4 mg/mL standard of THC (CON775) (equivalent to 40% THC in a plant) and analyze on both GC-MS 1 and 4.

Part 4 (9/16/19)

- Prepare a 0.1 mg/mL solution of CBC, CBD, ∆8-THC, ∆9-THC, CBG and CBN (CON776) and analyze on both GC-MS 1 and 4.

Step 2: Precision at the Decision Point

Analyst: Amber Kohl (AK) and Ilene Alford (IKA)
9/23/19-9/27/19

General Outline of Validation Step:

- Prepare a 0.05 mg/mL, 0.1 mg/mL and 0.15 mg/mL THC standard (CON780-CON782 and CON785-CON796) each day for five days and analyze in triplicate on GC-MS 1.

Step 3: Precision (Reproducibility)

Analyst: Amber Kohl (AK)
9/18/19 & 9/20/19

General Outline of Validation Step:

- Weigh one marijuana case sample (50 mg (±1 mg) each) ten times by crumbling with gloved hands onto weighing paper (seeds, stems, stalks or roots are not included), place into labeled test tubes and record the weight.
- Weigh one hemp case sample (50 mg (±1 mg) each) by cutting up into small pieces with scissors onto weighing paper and place into a labeled test tube (seeds, stems, stalks or roots are not included).
- One extraction blank is also included.
- Dissolve each sample in 5 mL of internal standard solution (ISTD215), invert once and sonicate for at least 10 minutes.

- Invert the test tubes to mix the solution, transfer approximately 1 mL of the solution into an autosampler vial and analyze on both GC-MS 1 and 4.

Step 4: Trueness

Analyst: Amber Kohl (AK)
9/26/19

General Outline of Validation Step:

- Add 900 µL of methanol containing the internal standard (ISTD215) using a calibrated 100-1000 µL pipette to an autosampler vial.
- Using a calibrated 10-100 µL pipette, add 100 µL of certified THC reference material (CON792).
- Weigh VAL-01, VAL-02, VAL-03 and VAL-04 (50 mg (±1 mg) each) in duplicate by crumbling with gloved hands onto weighing paper (seeds, stems, stalks or roots are not included), place into labeled test tubes and record the weight.
- Weigh a hemp sample (50 mg (±1 mg) each) in duplicate by cutting up into small pieces with scissors onto weighing paper (seeds, stems, stalks or roots are not included), place into labeled test tubes and record the weight.
- One extraction blank is also included.
- Dissolve each sample in 5 mL of internal standard solution (ISTD215), invert once and sonicate for at least 10 minutes.
- Invert the test tubes to mix the solution, transfer approximately 1 mL of the solution into an autosampler vial and analyze on GC-MS 1.

Step 5: Limit of Detection

Analyst: Amber Kohl (AK)
9/13/19

General Outline of Validation Step:

1. Make a 0.1 mg/mL THC positive control in ISTD215 and analyze by GC-MS (CON774).

Step 6: Ruggedness/Robustness

Analyst: Amber Kohl (AK), Marcus Warner (MW), Diana Lawrence (DL), Kelvin Morales (KMC), Steven Williams (SW), Ilene Alford (IKA)
9/13/19-10/16/19

General Outline of Validation Step:
Competency Testing

- Add 900 µL of methanol containing the internal standard (ISTD215) using a 100-1000 µL calibrated pipette to an autosampler vial.

- Using a calibrated 10-100 µL pipette, add 100 µL of certified THC reference material (CON799-802 and 804).
- Weigh an unknown sample (50 mg (±1 mg) each) in duplicate using weighing paper (seeds, stems, stalks or roots are not included), place into labeled test tubes and record the weight.
- Weigh a hemp sample (50 mg (±1 mg) each) in duplicate by cutting up into small pieces with scissors onto weighing paper (seeds, stems, stalks or roots are not included), place into labeled test tubes and record the weight.
- One extraction blank is also included.
- Dissolve each sample in 5 mL of internal standard solution (ISTD215), invert once and sonicate for at least 10 minutes.
- Invert the test tubes to mix the solution, transfer approximately 1 mL of the solution into an autosampler vial and analyze on GC-MS 1.

Step 7: Carryover

Analyst: Amber Kohl (AK)
9/16/19

General Outline of Validation Step:

1. Make a 4 mg/mL solution of THC in ISTD215 (equivalent to 40% THC in a plant) and analyze in triplicate on GC-MS 1 and 4 (CON775).
2. Run an IS blank sample after each injection.

Step 8: Extract Stability

Analyst: Amber Kohl (AK)
9/23/19-9/27/19

General Outline of Validation Step:

1. Extract three different marijuana samples and one hemp sample.
2. Combine the extracts of the marijuana replicates and then divide into five different vials.
3. Divide the hemp extract into 5 different vials.
4. Inject a vial of the combined marijuana extract and the hemp extract in triplicate on day 0.
5. Store the other vials on the instrument and re-inject on each subsequent day for at least three days in triplicate.
6. The response of each analyte must be within ± 20% of the response from day 0. If the response falls outside this range then the extract stability of the analyte has been exceeded.

6. Results

Step 1: Selectivity/Specificity

Five marijuana samples and one hemp sample were extracted and analyzed by GC-MS using the THCSCREN method to evaluate the selectivity of the method through an interference study. The

aim was to show there was no interference between THC and the IS from other compounds commonly found in Cannabis. No interferences at the retention time for THC or the IS were noted after analysis of the marijuana and hemp samples. In addition, when Step 4 (Trueness) was performed, there were no interferences found at the retention time for THC or the IS in any of the samples. There was also no interference between the retention time of the internal standard and the retention time of THC observed after analysis of a 0.1 mg/mL positive control (Table 1). A single peak with clear, non-splitting apex was observed for both THC and the IS and there was no fronting or tailing.

Table 1. Results for Step 1, Part 2 - Interference Study Between THC and the IS.

Instrument	THC Retention Time (min)	IS Retention Time (min)
GC-MS 1	3.03	4.06
GC-MS 4	3.40	4.15

Since the marijuana strain with one of the highest THC concentrations contains approximately 34% THC based on the dry weight of the plant[6], a 4 mg/mL solution (equivalent to 40% THC (w/w) in the plant) was analyzed to demonstrate that there was no interference with the IS from a high concentration of THC. The results demonstrated no interferences between the THC and the internal standard. Retention time differences between THC and the IS for this step were 1.01 and 0.69 min for GC-MS 1 and 4, respectively.

A selectivity mix containing five other compounds commonly found in Cannabis showed no interferences at the retention time for THC or the IS from these similar cannabinoids. The resolution between the closest eluting cannabinoid to THC and the IS was greater than 1. The compound with the closest retention time to THC was delta-8-THC (GC-MS 1 resolution = 1.51, GC-MS 4 resolution = 1.37). The compound with the closest retention time to the IS was CBN (GC-MS 1 resolution = 6.73, GC-MS 4 resolution = 4.22).

Conclusion: The method is specific for the target analyte (THC) and internal standard (testosterone) studied.

Step 2: Precision at the Decision Point

Tetrahydrocannabinol was evaluated for precision at the decision concentration of 0.1 mg/mL. Three different THC standards were prepared at the following concentrations: 0.05 mg/mL (50% below the decision point), 0.1 mg/mL (decision point) and 0.15 mg/mL (50% above the decision point). Each of the standards were analyzed in triplicate on five separate days. The results are displayed in Table 2.

Table 2. Results for Step 2 - Precision at the Decision Point.

0.05 mg/mL THC									
		THC:IS Area Ratios							
Injection #	Compound	Day 1	Day 2	Day 3	Day 4	Day 5	Grand Mean	Std Dev	Total THC:IS Area Ratio CV (%)
1	THC	0.721	0.990	0.786	0.645	0.644	0.787	0.134	17.0
2	THC	0.720	1.006	0.849	0.654	0.709			
3	THC	0.742	1.027	0.900	0.711	0.703			
0.10 mg/mL THC									
		THC:IS Area Ratios							
Injection #	Compound	Day 1	Day 2	Day 3	Day 4	Day 5	Grand Mean	Std Dev	Total THC:IS Area Ratio CV (%)
1	THC	2.276	2.363	2.036	1.832	1.608	2.005	0.271	13.5
2	THC	2.214	2.330	2.064	1.730	1.606			
3	THC	2.184	2.275	2.033	1.869	1.649			
0.15 mg/mL THC									
		THC:IS Area Ratios							
Injection #	Compound	Day 1	Day 2	Day 3	Day 4	Day 5	Grand Mean	Std Dev	Total THC:IS Area Ratio CV (%)
1	THC	3.468	3.763	3.223	3.030	2.973	3.293	0.283	8.6
2	THC	3.428	3.682	3.265	3.220	2.937			
3	THC	3.434	3.676	3.340	3.086	2.870			

The grand mean result for the THC:IS ratios at the 0.1 mg/mL decision point concentration was 2.005. Two standard deviations for the THC standards at 0.05 mg/mL and 0.15 mg/mL were 0.268 and 0.566, respectively. The standard deviation of the grand mean plus or minus two standard deviations for the THC standards at 0.05 mg/mL (0.519-1.055) and 0.15 mg/mL (2.727-3.859) did not overlap with the 2.005 mean value for the decision point concentration. The %CV for the 0.05, 0.10, and 0.15 ng/mL concentrations were 17.0, 13.5 and 8.6%, respectively; below the requirement to not exceed 20%.

Relative retention times for THC for the 0.1 mg/mL standard were also calculated for the fifteen injections. The %CV for the fifteen injections was 0.03%. The percentage difference in relative retention time between each injection and the average value obtained from all injections for the triplicate analysis of the standard over the five days was <0.05%. The absolute differences in the retention times for THC for these fifteen injections were all within ±0.04 minutes.

Conclusion: The method demonstrated acceptable precision at the decision point with all %CVs within 17% evaluated with three replicates of 0.05 mg/mL, 0.10 mg/mL and 0.15 mg/mL run on five separate days. The mean of the decision point and the mean +/- 2SD of the 0.15 mg/mL and the 0.05 mg/mL did not overlap. The relative retention time for THC displayed stability over fifteen injections within a five day period.

Step 3: Precision (Reproducibility)

In order to evaluate reproducibility, one marijuana case sample and one hemp sample were extracted ten times and then analyzed on both GC-MS 1 and GC-MS 4. On GC-MS 1, the %CV for

the THC:IS area ratios for the marijuana case sample analyzed was 7.0%. The THC:IS area ratios for the hemp sample analyzed was 13.2%. The higher %CV for the hemp compared to the marijuana is presumably due to the much lower concentration of THC in the hemp sample giving rise to more variability in the peak areas of those samples. GC-MS 4 showed THC:IS ratios of 6.8% and 25.5%, for marijuana and hemp, respectively. The lower sensitivity of GC-MS 4 resulted in the THC:IS peak areas varying greatly. Therefore, GC-MS 4 was deemed not suitable to use for this method.

Conclusion: GC-MS 1 showed %CV values well below the 20% acceptable criteria using both marijuana and hemp. GC-MS 4 displayed a value greater than 20% for the lower THC concentrations found in hemp and will not be used for this method.

Step 4: Trueness

Five Cannabis samples of known concentration were extracted and analyzed in duplicate and the THC concentration compared to known quantitative results for the samples. One of the samples was a hemp sample obtained from a hemp distributor. This sample had a certificate of analysis stating the total THC% was 0.450%. The four Cannabis samples were prepared and obtained from the Pinellas County Crime Laboratory (*Note: Validation Sample 5 is only listed for reference was not analyzed as part of this step):

1. Validation Sample 1 (Val-01) – Known illicit Cannabis (analyzed by an accredited laboratory to determine total THC). Dried and ground/homogenized in bead a ruptor prior to analysis. % total THC = 17.672%.
2. Validation Sample 2 (Val-02) – Combination of 1 part Val-01 and 10 parts Val-05; dried and ground/homogenized in a bead ruptor and then analyzed by an accredited laboratory to determine total THC = 2.428%.
3. Validation Sample 3 (Val-03) - Combination of 2 parts Val-01 and 10-parts Val-05; dried and ground/homogenized in a bead ruptor and then analyzed by an accredited laboratory to determine total THC = 3.601%.
4. Validation Sample 4 (Val-04) - Combination of 3 parts Val-01 and 10-parts Val-05; dried and ground/homogenized in a bead ruptor and then analyzed by an accredited laboratory to determine total THC = 4.412%.
5. Validation Sample 5 (Val-05) – Hemp product dried and homogenized in a bead ruptor with known total THC of <0.3%.

Following analysis, the THC:IS area response ratio was calculated for each sample analyzed (Table 3). This value was then averaged between the two duplicate samples taken for each and the %CV was calculated for the duplicate samples. The %CV of the duplicate samples were 3.2%, 7.3%, 4.3%, 2.2 and 14.1%, respectively, which was below the requirement of the duplicate samples being within 20%.

Table 3. Results for Step 4 – Trueness.

Sample Name	Sample #	Amount Sampled (mg)	THC:IS Response Ratio	Average	Total CV (%)	Calculated THC Conc. (%)	Actual THC Conc. (%)
VAL-01	1	50.4	35.63	36.44	3.2	21.1	17.7
VAL-01	2	50.4	37.26				
VAL-02	1	49.3	4.05	3.85	7.3	2.2	2.4
VAL-02	2	50.0	3.65				
VAL-03	1	50.4	6.19	6.38	4.3	3.7	3.6
VAL-03	2	50.9	6.58				
VAL-04	1	49.8	8.11	7.99	2.2	4.6	4.4
VAL-04	2	50.9	7.87				
HEMP	1	50.9	1.10	1.00	14.1	0.58	0.45
HEMP	2	50.7	0.90				

The following equation was utilized to calculate the THC:IS area ratios for the samples:

THC:IS Area Sample Response Ratio = [Sample THC (area)/IS (area)] x [50 mg/Amount sampled]

The THC:IS area ratio for the positive control analyzed in Step 4 was 1.73. The peak area ratios for VAL-01, VAL-02, VAL-03 and VAL-04 were all greater than the corresponding positive control and had known total THC concentrations of greater than 1%. The peak area ratios for the hemp sample was less than the positive control and had a known total THC concentrations of less than 1%. Most calculated THC values for the samples were particularly close to the actual known THC concentration in the plant sample. The calculated THC concentrations in tables 3 and 4 are an estimate based on the assumption that there is a directly proportional relationship between the THC:IS ratio and concentration and were used to estimate the concentration for evaluation purposes only. This method is validated to demonstrate that the sample contains at least 1% total delta-9 THC and is not used to determine the actual THC concentration. The slight decrease in concentration (0.0675 mg/mL) of the internal standard in the positive control was noted, but creates a more conservative threshold still demonstrating that the sample contains at *least 1%* THC.

Conclusion: Based on this testing, the method is demonstrated to be suitable for estimating if the level of THC in a sample of Cannabis plant material is above or below 1% (w/w).

Step 5: Limit of Detection (LOD)

The decision point for a method is defined as an administratively defined cutoff or concentration that is at or above the method's limit of detection. Therefore, the analytical LOD for this method is the decision point concentration of 0.1 mg/mL and is used to discriminate between positive and negative results. Both the THC and the IS had good chromatographic peak shape at the LOD. Tetrahydrocannabinol demonstrated signal-to-noise ratios greater than 10:1 on both GC-MS 1 and GC-MS 4 (S/N = 202 and 615, respectively). The instrumental LOD for THC where the S/N was greater than 10 was previously done on both GC-MS1 and GC-MS 4 and was determined to be 0.025 mg/mL and 0.05 mg/mL, respectively.

Conclusion: The LOD for the method was defined as 0.1 mg/mL and demonstrated good peak shape for THC and the IS and suitable signal-to-noise for THC.

Step 6: Ruggedness/Robustness

Validation studies were performed over multiple days and demonstrated repeatable results. All five analysts in the Chemistry Unit were competency tested and obtained acceptable results after analyzing one sample above the decision point and one sample below the decision point (Table 4). The competency testing took place over a 2 week period. Each analyst prepared a THC positive control on the day the unknown and hemp sample were analyzed from the same vial of THC certified reference material. The CV% for the THC:IS area ratios for all of the positive controls prepared was 5.4%. Therefore, the certified THC reference material was stable after opening over this 2 week period. For all analysts, the THC:IS area ratios for all unknown samples were greater than that of the corresponding positive controls, whereas, the hemp samples that were analyzed had THC:IS area ratios less than that of the positive control. The calculated THC concentrations were consistent with the known total THC concentration in the samples.

Table 4. Results for Step 6 – Competency Testing.

Analyst	Replicate #	Sample Name	THC:IS Response Ratio	Average	Total CV (%)	Calculated THC Conc. (%)	Actual THC Conc. (%)
MW	1	VAL-02	4.54	4.36	5.8	1.9	2.4
	2	VAL-02	4.18				
	1	Hemp	1.03	1.00	5.0	0.44	0.45
	2	Hemp	0.96				
DL	1	VAL-04	8.44	8.51	1.2	3.8	4.4
	2	VAL-04	8.59				
	1	Hemp	0.76	0.80	7.6	0.36	0.45
	2	Hemp	0.84				
KM	1	VAL-03	6.86	6.95	1.8	3.3	3.6
	2	VAL-03	7.04				
	1	Hemp	0.82	0.82	0.5	0.38	0.45
	2	Hemp	0.81				
SJW	1	VAL-02	3.74	4.20	15.4	1.7	2.4
	2	VAL-02	4.66				
	1	Hemp	0.78	0.80	3.2	0.33	0.45
	2	Hemp	0.82				
IKA	1	VAL-03	8.64	8.68	0.7	3.6	3.6
	2	VAL-03	8.72				
	1	Hemp	0.89	0.88	2.1	0.37	0.45
	2	Hemp	0.86				
THC:IS Area Ratios for THC Positive Controls: MW = 2.28, DL = 2.22, KM = 2.12, SJW = 2.43, IKA = 2.38							

Conclusion: Overall, the method demonstrated acceptable robustness and yielded repeatable results. All analysts in the Chemistry Unit are competent in the execution of the method.

Step 7: Carryover

The lack of carryover was determined by triplicate analyses on both GC-MS 1 and 4. A blank IS sample was THC free when run after a standard prepared at a concentration 4 mg/mL (equivalent to 40% THC dry weight in the plant). Throughout the validation, IS blanks were routinely run between Cannabis samples and no carryover was observed.

Conclusion: The method demonstrated a lack of carryover up to a concentration of 4 mg/mL for THC. Internal standard blanks will be run prior to each case sample to demonstrate that carryover did not occur.

Step 8: Extract Stability

Three different marijuana samples and one hemp sample were extracted. The marijuana extracts were combined and then divided into five different vials. One vial of marijuana extract and the hemp extract were injected in triplicate on day 0. The other vials were stored on the instrument and re-injected on days 1, 2, 3 and 4 in triplicate. The THC response remained within ±20% of the response from day 0 (Table 5). The %CV for the THC:IS area ratio for triplicate injections was averaged for each day and then compared to the average of the triplicate injections at time 0. After 4 days from the date of extraction, the %CV was 13.6% for marijuana and 5.7% for hemp. Therefore, the extract stability of THC was not exceeded and was confirmed to be stable for at least 4 days after the date of extraction.

Table 5. Results for Step 8 – Extract Stability.

Marijuana										
	Peak Areas					THC:IS Area Ratios				
Injection #	Time 0	Day 1	Day 2	Day 3	Day 4	Time 0	Day 1	Day 2	Day 3	Day 4
1	9126121	8955618	9146389	9258321	9376018	58.531	55.213	49.504	47.483	49.419
2	9527766	9381641	9526606	9503186	9660651	57.977	53.392	50.121	51.402	49.123
3	9731456	9655482	9603297	9716153	9886909	54.840	49.651	48.146	46.346	42.732
					Average	57.116	52.752	49.257	48.410	47.091
					%CV	-	5.6	10.4	11.7	13.6
Hemp										
	Peak Areas					THC:IS Area Ratios				
Injection #	Time 0	Day 1	Day 2	Day 3	Day 4	Time 0	Day 1	Day 2	Day 3	Day 4
1	147709	149545	160571	164175	179315	0.876	0.831	0.823	0.814	0.838
2	147167	147480	152816	154393	164891	0.852	0.801	0.744	0.754	0.764
3	148334	144258	152985	154653	164885	0.842	0.770	0.765	0.739	0.768
					Average	0.857	0.801	0.777	0.769	0.790
					%CV	-	4.8	6.9	7.6	5.7

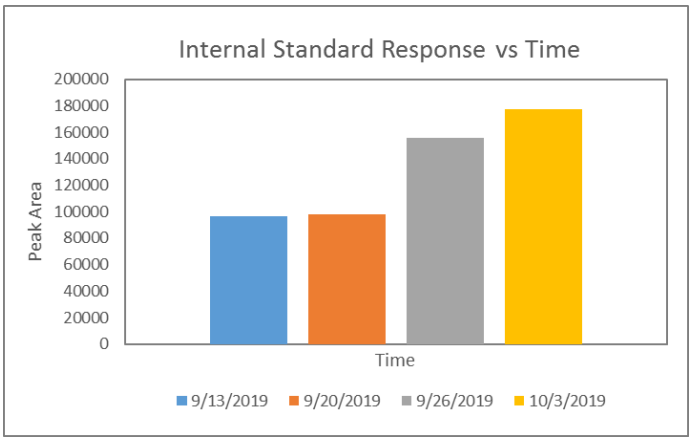
Conclusion: Extracts were confirmed to be stable for at least 4 days after the day of extraction for THC. Extracts may be analyzed for at least 4 days after the date of extraction for THC.

Additional Performance Criteria Evaluated: Stability of the Internal Standard

Throughout this validation, internal standard blanks (testosterone) were routinely analyzed between samples that were run on GC-MS 1. Data from area responses of these internal standard blank samples was evaluated for three blanks run on the same day each week of the validation. The triplicate samples were then averaged for each day and plotted as a function of time (Plot 1). This was to determine the stability of the internal standard. According to the plot, there was

no decrease in area response of testosterone over time. The increase in response is presumably due to instrumental fluctuations in response over time.

Plot 1. Internal Standard Stability



Conclusion: There was no decrease in testosterone response over time. Testosterone is stable and suitable for use as the internal standard.

7. References

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