Differentiation of Hemp from Marihuana Using a Qualitative Decision-Point Assay

-Preface-

In late February 2020, the National Institute on Drug Abuse (Drug Supply Program) provided marihuana plant material for a collaborative study between Sam Houston State University (SHSU), Houston Forensic Science Center (HFSC), Harris County Institute of Forensic Sciences (HCIFS) and the Texas Department of Public Safety (DPS) Crime Laboratory Service. The US Drug Enforcement Administration (DEA) was the first to deploy a "decision point" gas chromatography/mass spectrometry (GC/MS) assay as part of their analytical scheme to differentiate hemp from marihuana. This approach, which has since been successfully implemented in other laboratories, was the subject of an inter-laboratory study, facilitated by Sam Houston State University and the Texas Forensic Science Commission.

This document describes the analytical protocol and performance of the assay among participating laboratories. The analytical protocol presented here represents the conditions under which satisfactory performance was achieved at all sites. **The information presented as part of this collaborative study does not supplant the need for there to be a full, independent, and rigorous validation if the method is deployed elsewhere due to inter-laboratory differences.**

This document summarizes the analytical protocol rather than a standard operating procedure. Results of the laboratory's own method validation will impact the final standard operating procedure, which typically includes the protocol in addition to sampling, specific quality assurance and reporting guidance, and therefore varies between laboratories.

Each of the collaborating laboratories selected an administrative threshold in plant material of 1% Δ9-tetrahydrocannabinol (Δ9-THC) by weight, rather than the 0.3% established by law. In addition to this safeguard, **the analytical approach is likely to *underestimate* the total Δ9-THC due to incomplete extraction efficiencies and decarboxylation rates**. It should be noted that this conservative approach is designed to prevent false positive results and increase specificity at the expense of sensitivity. Assay specificity (the ability to correctly identify a known negative sample) was 100% across all sites, and assay sensitivity (the ability to correctly identify a known positive sample) was 94%. Laboratories should consider these variables when establishing the appropriate administrative threshold to be used in their laboratory.

Analytical Protocol for the Qualitative Identification of Marihuana using GC/MS

1. Introduction

This GC/MS procedure is used to distinguish between potential hemp as defined per Agriculture Code 121.001 and marihuana as defined per the Texas HSC 481.002.26 as of June 10, 2019. An administrative threshold of 1% Δ9-tetrahydrocannabinol (Δ9-THC) by weight is established for reporting purposes.

2. Scope

This method:

- Identifies marihuana
- Applies to plant material with individual net weights above 50 mg
- Does not apply to seeds, oils, edibles, products, roots, or other substances

3. Analytical Technique

Cannabinoids are extracted from plant material using organic solvent. Extracts are analyzed using gas chromatography/mass spectrometry (GC/MS) using a decision-point ratio equivalent to 1% Δ9-THC (w/w). Total Δ9-THC is determined following the heat-mediated decarboxylation of tetrahydrocannabinolic acid in the GC inlet.

4. Equipment/Materials/Reagents

1. Analytical balance
2. Volumetric glassware and tools
    a. Glass test tubes (10x75mm) or similar
    b. Autosampler vials with inserts and caps
    c. Volumetric pipettes (20-200µL; 200-1000 µL; 1-5mL)
    d. Volumetric flasks (Class A)
    e. Volumetric cylinder (1L, Class B; <1L, Class A)
    f. Glass Pasteur pipettes
    g. Syringe filters/filtration devices
3. Solvents
    a. Methanol (MeOH) (Analytical grade or higher)
4. Chemicals
    a. Δ9-THC analytical standard or certified reference material (CRM)
    b. Δ9-THC-D3 analytical standard or certified reference material (CRM)

5. Preparation of Reagents/Solutions/Standards

- **Methanol**
- **Δ9-tetrahydrocannabinol (THC) analytical reference standard or certified reference material (CRM) (1 mg/mL)**
  Purchase the THC analytical standard or CRM (1 mg/mL in MeOH) from an approved vendor. Store ampoules frozen until use.
- **Δ9-tetrahydrocannabinol-D3 (THC-D3) analytical reference standard or certified reference material (CRM) (1 mg/mL or 0.1 mg/mL)**
  Purchase the THC-D3 analytical standard or CRM (1 mg/mL or 0.1 mg/mL in MeOH) from an approved vendor. Store ampoules frozen until use.
- **THC Standard Solution (0.05 mg/mL)**
  Using a volumetric pipette, transfer 500 µL of the THC analytical reference standard or CRM (1 mg/mL) into a 10 mL volumetric flask (Class A). Bring to volume with MeOH. Store frozen until use. Equivalent dilutions should be performed if solutions are prepared on a different scale.
- **THC-D3 Internal Standard Solution (ISS) (0.1 mg/mL)**
  The ISS consists of 0.1 mg/mL of THC-D3 in MeOH which may be purchased as an analytical reference standard or certified reference material and used directly. If using a 1 mg/ml standard, prepare the ISS by diluting analytical reference standard or certified reference material (CRM). Using a volumetric pipette, transfer 1000 µL of THC-D3 (1 mg/mL) into a 10 mL volumetric flask (Class A) and bring to volume with MeOH. Store in the freezer until use. Equivalent dilutions should be performed if solutions are prepared on a different scale.
- **Cannabinoid Mix**
  A qualitative cannabinoid mix may be used to identify compounds other than Δ9-THC.

6. Standards and Controls

- **Negative Control**

  Mix equal volumes of Internal Standard Solution (ISS) (0.1 mg/mL THC-D3) with MeOH. For example, using a volumetric pipette, transfer 50 µL of ISS and 50 µL MeOH into an autosampler vial. Vortex mix and tightly cap. Prepare this negative control with each batch of unknown samples. The negative control, positive control and unknowns (case samples) must be prepared using the same batch of ISS.

- **Positive Control (Decision-Point Control) and Secondary Control**

  Mix equal volumes of THC Standard Solution (0.05 mg/mL) with Internal Standard Solution (ISS) (0.1 mg/mL THC-D3). For example, using a volumetric pipette, transfer 50 µL of THC Standard Solution and 50 µL ISS into an autosampler vial. Vortex mix and tightly cap. Prepare this positive control with each batch of unknown samples. The negative control, positive control and unknowns must be prepared using the same batch of ISS. A second positive control prepared from a different lot number (or vendor) should also be prepared. This is referred to as the Secondary Control.

7. Procedure

1. Using an analytical balance, transfer 50 mg (± 0.5 mg) of dry plant material into a glass test tube. Sample from buds and leaves of the plant. The bud/leaf sample may be broken-up manually with gloved fingers if necessary.

2. Using a 5 mL volumetric pipette, add 2 x 5.0 mL MeOH for a total of 10 mL. Vortex 10 seconds. Let stand 5 minutes and vortex an additional 10 seconds. If particulate material is present, samples can be filtered through a Pasteur pipette containing cotton, a syringe filter, or similar device.

3. Mix equal volumes of sample extract (above) with Internal Standard Solution. For example, using a volumetric pipette, transfer 50 µL of sample and 50 µL ISS into an autosampler vial. Vortex mix and tightly cap. The sample must be prepared using the same batch of ISS as the positive and negative controls.

4. Prepare a negative control, positive control, and secondary control from the same ISS as the batch of unknown samples.

5. Inject 1 µL of the controls and unknown samples onto the GC/MS in the following order:

   Positive Control (Decision-Point Control)
   Secondary Control (from an independent vendor or lot number)
   Negative Control
   Unknown plant extracts
   Positive Control (Decision-Point Control) – reinjected at the end of the batch

   Inject methanol blanks in between the unknown plant extract samples using the instrumental conditions described below. Reinject the positive control (Decision-Point Control) after every ten plant extracts.

6. A schematic for the extraction is shown in the appendix.

8. Instrumental Analysis

Plant extracts and controls are analyzed using an acquisition method that can separate natural and synthetic cannabinoids. The split ratio should be modified to adjust for sensitivity and instrument performance. Data is acquired in selected ion monitoring (SIM) and full scan mode. The decision-point value is determined using SIM data for THC and the IS (THC-D3) (using the RTE integrator). Full scan data facilitates the identification of additional cannabinoids or compounds, if desired.

Method Parameters
Instrument: Agilent GC/MS
GC Column Type: DB-5 (30 m x 0.25 mm x 0.25 µm)
Inlet Temperature: 250°C
Injection Mode: Split

Spilt Ratio: 100:1 to 20:1
Injection volume: 1 µL
Solvent Rinse: Methanol (A & B)
Injector Rinse: Pre-injection (2A, 2B); post-injection (10A, 10B)
Carrier Gas and Flow: Helium, 1.5 mL/min
Control Mode: Constant flow
Oven Program: 200 °C initial temperature ramped to 235°C at 15 °C /min; hold for 7 min; ramp to 290°C at 30 °C/min
Run Time: 12.17 min
Ionization Mode: Electron ionization
Solvent Delay: 2 min
Scan Range: *m/z* 40-550 (full scan)
SIM Ions: THC: *m/z* 314, 231, 271 (40 ms dwell time); THC-D3: *m/z* 317, 234, 274 (40 ms dwell time)
MS Source Temperature: 230°C
MS Quadruple Temperature: 150°C
Transfer Line Temperature: 280°C
Tune Type: stune

9. Data Interpretation

1. Differentiation of hemp from marijuana using a 1% administrative threshold is performed using data acquired in SIM mode. Retention times and ion ratios for THC and THC-D3 from the initial positive control are used to establish acceptance criteria. Retention times for THC and THC-D3 for all subsequent controls (including the secondary control) within a batch shall be within 1%, and ion ratios shall be within ±20% of the established values for the initial positive control.

2. The relative peak area (RPA) of THC/IS (THC/THC-D3) is determined for the initial positive control and for the secondary control. These values must be within ±20% of each other for the batch to be acceptable.

3. The relative peak area (RPA) of THC/IS (THC/THC-D3) is determined for each positive control within a batch, and the average RPA is determined from these values. The individual RPA for all positive controls must be within ±20% of the average RPA for the batch to be acceptable.

4. If the acceptance criteria for the controls within a batch are met, then the plant extracts will be evaluated. To be acceptable, retention times of THC and THC-D3 for plant extracts shall be within 1% and ion ratios shall be within ±20% of the established values for the initial positive control.

5. The RPA for the initial and reinjected positive controls are compared, and the positive control with the highest RPA is used to establish a decision-point ratio (DPR) by normalizing all of the RPA values within the batch to this value. The positive control with the highest RPA will therefore always have a DPR value of 1.0.

6. Plant extracts with decision-point ratios below 1.0 (or when acceptance criteria for the plant extracts are not met) do not meet the administrative threshold for the identification of marihuana.

7. Plant extracts with decision-point ratios at or above 1.0 meet the administrative threshold for the identification of marihuana.

10. Dilution Integrity

Plant extracts containing high concentrations of Δ9-THC may require dilution (post-extraction) to prevent overloaded peaks or sub-optimal chromatography. When plant extracts do not produce results that meet acceptance criteria but have a DPR value above 1.0, the extract may be diluted and reinjected if necessary.

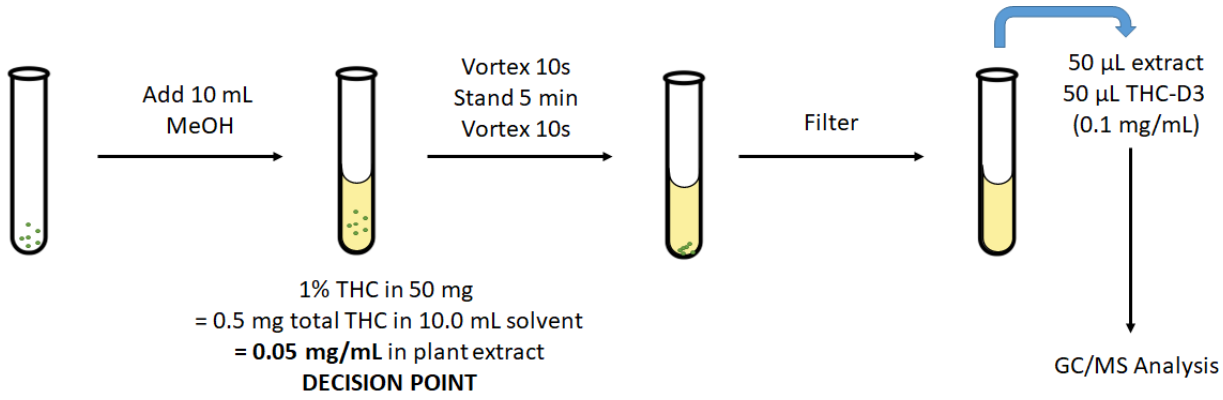
1. Perform the appropriate dilution using a volumetric pipette. For example, to achieve a 10-fold dilution, combine 50 µL of plant extract with 450 µL of MeOH and mix. Other dilution factors may be used. Five and ten-fold dilutions are generally sufficient.

2. Mix equal volumes of the diluted extract with ISS as described earlier. For example, using a volumetric pipette, transfer 50 µL of the diluted extract and 50 µL ISS into an autosampler vial. Vortex mix and tightly cap. The sample must be prepared using the same batch of ISS as the positive and negative controls.

3. Diluted plant extracts with decision-point ratios below 1.0 (or when acceptance criteria for the plant extracts are not met) do not meet the administrative threshold for the identification of marihuana.

4. Diluted plant extracts with decision-point ratios at or above 1.0 meet the administrative threshold for the identification of marihuana.

Appendix - Extraction Schematic



Summary of Interlaboratory Validation

1. Introduction

Gas chromatography/mass spectrometry (GC/MS) is used to distinguish between hemp (as defined in the Texas Agriculture Code 121.001) and marihuana (as defined in the Texas Health and Safety Code 481.002.26). An administrative threshold of 1% Δ9-tetrahydrocannabinol (Δ9-THC) is used for reporting purposes using a decision-point control. This document summarizes the results of an interlaboratory validation involving four sites using a 12 minute assay and a deuterated internal standard (Δ9-THC-D3 or simply THC-D3).

2. Scope

Assay performance was evaluated in terms of sensitivity, specificity, selectivity, detection limit, carryover, precision, accuracy, processed sample stability, range, interference from cannabidiol (CBD), and dilution integrity[1]. In-situ decarboxylation was also assessed.

3. Selectivity and Retention Time Stability

The selectivity of the method was evaluated using a variety of cannabinoids, including some synthetic analogs. Retention times for Δ9-THC, internal standard (THC-D3) and other compounds are shown in Table 1. A sample total ion chromatogram (TIC) is shown in Figure 1. PCP is included for reference. Nicotine elutes during the solvent delay under these method conditions. Intra-assay retention time stability was evaluated using 10 replicate injections of a mixed standard solution in a single day (n=10). Inter-assay retention time stability was evaluated using a single injection each day for ten days (n=10) (Table 2).

Table 1. Selectivity.

	SHSU		HCIFS		HFSC		DPS	
	RT (min)	RRT	RT (min)	RRT	RT (min)	RRT	RT (min)	RRT
THC-D3	9.598	1.000	9.178	1.000	8.282	1.000	9.079	1.000
Cannabidivarin (CBDV)	5.108	0.533	4.900	0.533	4.559	0.550	4.992	0.542
Cannabichromevarin (CBCV)	5.246	0.546	5.032	0.548	4.626	0.559	4.999	0.551
Cannabicitran (CBT)	5.934	0.618	-	-	5.177	0.625	-	-
Tetrahydrocannabivarin (THCV)	6.233	0.649	5.961	0.649	5.486	0.662	5.955	0.656
Cannabivarin (CBV)	6.404	0.667	6.135	0.668	5.703	0.689	-	-
Cannabicyclol (CBL)	7.017	0.731	6.719	0.732	6.085	0.735	6.634	0.731
Cannabidiol (CBD)	7.748	0.808	7.412	0.807	6.787	0.819	7.430	0.818
Cannabichromene (CBC)	7.898	0.823	7.560	0.823	6.787	0.819	7.430	0.818
Exo-THC	8.713	0.908	8.332	0.907	7.547	0.911	-	-
Δ8-Tetrahydrocannabinol (Δ8-THC)	9.173	0.956	8.770	0.995	7.919	0.956	8.693	0.957

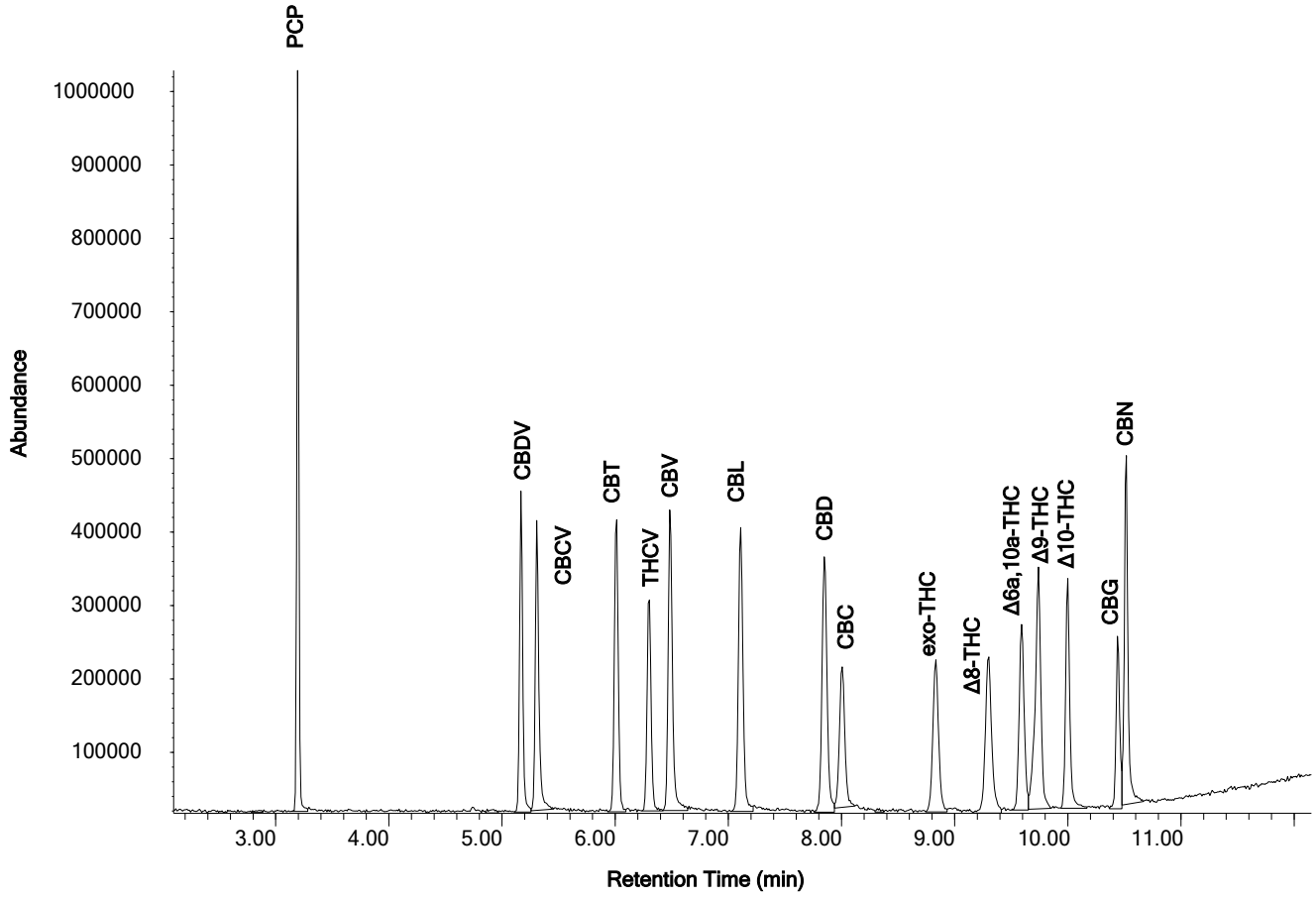
1. ASTM E2549 Standard Practice for Validation of Seized Drug Analytical Methods.

Δ6a,10a-Tetrahydrocannabinol (Δ6a,10a-THC)	9.482	0.988	9.073	0.988	8.216	0.992	9.024	0.994
Δ9-Tetrahydrocannabinol (Δ9-THC)	9.647	1.005	9.267	1.009	8.336	1.007	9.157	1.009
Δ10-Tetrahydrocannabinol (Δ10-THC)	9.912	1.033	9.607	1.046	8.687	1.049	9.537	1.050
Cannabigerol (CBG)	10.378	1.080	10.157	1.106	9.398	1.135	10.059	1.108
Cannabinol (CBN)	10.449	1.089	10.237	1.115	9.619	1.161	10.209	1.124

Table 2. Retention time stability for Δ9-THC and other cannabinoids (n=10).

Site	**Δ9-THC**				**Other Cannabinoids**			
	Intra-Assay		Inter-Assay		Intra-Assay		Inter-Assay	
	Max RT Difference (Mins)	RT CVs (%) n=10	Max RT Difference (Mins)	RT CVs (%) n=10	Max RT Difference (Mins)	RT CVs (%) n=10	Max RT Difference (Mins)	RT CVs (%) n=10
SHSU	0.010	0.03	0.006	0.03	0.020	0-0.07	0.020	0-0.10
HCIFS	0.008	0.04	0.072	0.31	0.009	0-0.06	0.032	0-0.30
HFSC	0.000	0.00	0.010	0.06	0.020	0-0.10	0.029	0-0.09
DPS	0.010	0.06	0.020	0.05	0.020	0.05	0.020	0.04-0.10

Figure 1. Representative TIC using the long method (provided by SHSU).



4. Limit of Detection and Linearity of Detector Response

The limit of detection (LOD) and linearity of the detector response was determined using serial dilutions of a Δ9-THC standard (prepared from a certified reference material) using replicate injections (n=4) at each concentration. The LOD was defined as the lowest concentration of Δ9-THC to produce a signal-to-noise (S/N) ratio of 3 or more, RT within 1% of the expected value, and ion ratios within ±20% (Table 3). Linearity in detector response, coefficients of determination (R^2) and CVs (n=4) are summarized in Table 4.

Table 3. Limit of detection.

Site	LOD in Extract (mg/mL)	LOD (% Δ9-THC by weight)
SHSU	0.015[2]	0.3%
HCIFS	0.0078	0.15%
HFSC	0.0078	0.15%
DPS	0.015	0.3%

Table 4. Linearity in detector response.

Site	Linear Detector Response (mg/mL in extract)	Linear Detector Response (% Δ9-THC by weight)	R^2	CV Range (%) n=4
SHSU	0.0156 – 0.50	0.3 – 10%	1.000	0.9 – 2.8
HCIFS	0.0078 – 0.25	0.15 – 5%	0.992	0.8 – 2.5
HFSC	0.0078 – 0.50	0.15 – 10%	0.995	0.6 – 2.8
DPS	0.0078 – 0.25	0.15 – 5%	0.996	0.5 – 3.2

5. Carryover

Carryover was assessed using methanol blanks injected after increasingly higher concentrations of Δ9-THC. The sequence of high Δ9-THC followed by methanol blanks was performed five times. The highest concentration of Δ9-THC to produce no carryover (*i.e.* no reportable Δ9-THC) in any of the methanol blanks is summarized in Table 5.

Carryover was also assessed using a sequence of high concentration of Δ9-THC, methanol blank, followed by the decision-point control (0.05 mg/mL Δ9-THC in methanol, or 1% Δ9-THC by weight of plant material). The highest concentration of Δ9-THC to produce a decision-point ratio (relative peak areas of Δ9-THC/IS normalized to the initial decision-point control) within 20% of the initial decision-point control (administrative threshold) is summarized in Table 6.

[2] LOD determined in (placebo) plant matrix.

Table 5. Carryover – evaluated using methanol blanks.

Site	Highest Δ-9 THC Concentration with no Carryover (In extract)	Highest Δ9-THC Concentration with no Carryover (Weight of plant material)
SHSU	2.5 mg/mL[3]	50%
HCIFS	5.0 mg/mL	100%
HFSC	4.0 mg/mL	80%
DPS	5.0 mg/mL	100%

Table 6. Carryover – evaluated using the decision-point control.

Site	Highest Δ-9 THC Concentration with no Carryover (In Extract)	Highest Δ9-THC Concentration with no Carryover (Weight of plant material)	Decision-Point Ratio
SHSU	2.5 mg/mL[4]	50%	1.02 – 1.04
HCIFS	5.0 mg/mL	100%	1.00 - 1.09
HFSC	4.5 mg/mL	90%	1.01 – 1.05
DPS	5.0 mg/ml	100%	1.02 – 1.05

6. Precision – Repeatability of the Decision-Point Control

Repeatability was established using the change in the relative peak area (RPA) of Δ9-THC/IS using ten independently prepared decision-point controls, each injected once (on the same day). The covariance (%CV) for the absolute peak areas (Δ9-THC and THC-D3) are also shown (Table 7).

Table 7. Repeatability at the decision-point.

Site	RPA CV (%) n=10	Δ9-THC Absolute Peak Area CV (%) n=10	THC-D3 Absolute Peak Area CV (%) n=10
SHSU	3.4%	6.1%	7.4%
HCIFS	1.9%	3.4%	2.8%
HFSC	3.7%	18.2%	14.9%
DPS	2.1%	12.8%	12.6%

[3] Highest concentration tested.
[4] Highest concentration tested.

7. Precision – Reproducibility of the Decision-Point Control

Reproducibility was assessed using the change in the relative peak areas (RPA) of Δ9-THC/IS over five days. A decision-point control (prepared fresh daily) was injected ten times daily over five days. Intra- (n=10) and inter-assay (n=50) CVs for the relative peak areas are summarized in Table 8.

Table 8. Reproducibility at the decision-point.

	RPA Intra-day CV (%) n=10	RPA Inter-assay CV (%) n=50
SHSU	0.7-1.7%	5.1%
HCIFS	1.3 - 2.6%	3.6%
HFSC	1.6 – 2.1%	3.5%
DPS	1.3 - 1.8%	2.4%

8. Accuracy

Accuracy of the method was established using fourteen *Cannabis sativa* plant matrices, each extracted five times (n=70 per site). Seven of the plant matrices contained Δ9-THC above the administrative threshold (1% by weight) and seven were below. Nine plants provided by the National Institute on Drug Abuse (Drug Supply Program) contained between 0.12-10.1% total Δ9-THC[5]. Five commercial hemp samples were also purchased, including CBD and CBG-rich samples. The chemical composition (as defined by the supplier) are summarized in Table 9.

Table 9. Chemical composition of *Cannabis sativa* plant matrices (P1-P7 and N1-N7).

Plant ID	**Source**	**Concentration (% by weight)**					**Comments**
		Total Δ9-THC	**Δ8-THC**	**CBD[6]**	**CBN[7]**	**CBG[8]**	
Above administrative threshold (P1-P7) – TRUE POSITIVE							
P1	NIDA	1.9 ± 0.06	ND	0.17	0.52	NK	Δ9-THC (0.25), THCA-A (1.3), THCA-B (0.39)
P2	NIDA	3.9 ± 0.12	ND	0.01	0.38	NK	Δ9-THC (0.61), THCA-A (3.1), THCA-B (0.21)
P3	NIDA	8.0 ± 0.63	ND	0.09	0.62	NK	Δ9-THC (0.76), THCA-A (6.6), THCA-B (0.68)
P4	NIDA	6.7 ± 0.26	ND	0.02	0.48	NK	Δ9-THC (0.87), THCA-A (5.6), THCA-B (0.28)

[5] Determined by GC analysis; Inclusive of Δ9-THC, THCA-A (tetrahydrocannabinolic acid A), THCA-B (tetrahydrocannabinolic acid B) as per data sheet.
[6] Reflects total CBD for NIDA plant material (GC analysis) and commercial hemp.
[7] Reflects total CBN for NIDA plant material (GC analysis).
[8] Reflects total CBG for commercial hemp.

P5	NIDA	10.1 ± 0.65	ND	0.04	0.89	NK	Δ9-THC (1.4), THCA-A (8.0), THCA-B (0.71)
P6	NIDA	2.4 ± 0.17	0.01	3.7	0.24	NK	Δ9-THC (0.27), THCA-A (2.0), THCA-B (0.15)
P7	NIDA	2.4 ± 0.17	0.01	3.8	0.25	NK	Δ9-THC (0.26), THCA-A (2.0), THCA-B (0.14)
Below administrative threshold (N1-N7) – TRUE NEGATIVE							
N1	NIDA	0.12 ± 0.02	0.01	3.3	0.03	NK	Δ9-THC (0.03), THCA-A (0.09)
N2	NIDA	0.37 ± 0.03	0.02	9.2	0.05	NK	Δ9-THC (0.08), THCA-A (0.29)
N3	Commercial hemp	<LOQ	<LOQ	21.8	<LOQ	0.67	Δ9-THC LOQ (0.116), THCA LOQ (0.116), Δ8-THC LOQ (0.058)
N4	Commercial hemp	<LOQ	<LOQ	18.3	<LOQ	0.51	Δ9-THC LOQ (0.255), THCA LOQ (0.255), Δ8-THC LOQ (0.128)
N5	Commercial hemp	<LOQ	<LOQ	14.8	<LOQ	0.59	Δ9-THC LOQ (0.119), THCA LOQ (0.119), Δ8-THC LOQ (0.060)
N6	Commercial hemp	0.21	<LOQ	<LOQ	<LOQ	19.1	Δ9-THC LOQ (0.253), THCA (0.241), total Δ9-THC = THCA*0.877, Δ8-THC LOQ (0.126), CBD LOQ (0.253), CBDA LOQ (0.253)
N7	Commercial hemp	0.21	<LOQ	<LOQ	<LOQ	17.3	Δ9-THC LOQ (0.251), THCA (0.240), total Δ9-THC = THCA*0.877, Δ8-THC LOQ (0.126), CBD LOQ (0.251), CBDA LOQ (0.251)

For the purpose of this validation, positive indicates a decision-point ratio at or above the administrative threshold (1% Δ9-THC by weight) (*i.e.* a decision-point ratio of 1.0 or above), while negative indicates a result that is below the administrative threshold (*i.e.* a decision-point ratio < 1.0). Qualitative results are summarized in Table 10.

True positive (TP), true negative (TN), false positive (FP) and false negative (FN) results are summarized in Table 11. No false positive results were observed. False negative results from some sites were obtained using one *Cannabis sativa* sample (P1) from NIDA. This plant contained 0.25% Δ9-THC, 1.3% THCA-A and 0.39% THCA-B (Table 9). False negative results (below the administrative threshold) may be attributed to incomplete decarboxylation rates (<100%) for the *in-situ* conversion

of tetrahydrocannabinolic acid (THCA) to THC during GC/MS analysis, and the decreased stability of THCA-A[9] (which predominated in P1) relative to THCA-B.

Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were evaluated as follows:

$$\text{Sensitivity} = TP/(TP+FN)$$

$$\text{Specificity} = TN/(FP+TN)$$

$$PPV = TP/(TP+FP)$$

$$NPV = TN/(FN+TN)$$

Assay specificity (the ability to correctly identify a known negative sample) was 100% for all sites. Assay sensitivity (the ability to correctly identify a known positive) was 94% for all sites combined (range 86-100%). Positive predictive values for all sites were 100% and negative predictive values were 95% (range 88-100%).

Table 10. Summary of qualitative results for *Cannabis sativa* extracts (P1-P7 and N1-N7).

Plant ID	Source	Total Δ9-THC	Result			
			SHSU	HCIFS	HFSC	DPS
Above administrative threshold (N1-N7)						
P1	NIDA	1.9	Positive (n=5)	Negative (n=5)	Positive (n=4) Negative (n=1)	Positive (n=3) Negative (n=2)
P2	NIDA	3.9	Positive (n=5)	Positive (n=5)	Positive (n=5)	Positive (n=5)
P3	NIDA	8.0	Positive (n=5)	Positive (n=5)	Positive (n=5)	Positive (n=5)
P4	NIDA	6.7	Positive (n=5)	Positive (n=5)	Positive (n=5)	Positive (n=5)
P5	NIDA	10.1	Positive (n=5)	Positive (n=5)	Positive (n=5)	Positive (n=5)
P6	NIDA	2.4	Positive (n=5)	Positive (n=5)	Positive (n=5)	Positive (n=5)
P7	NIDA	2.4	Positive (n=5)	Positive (n=5)	Positive (n=5)	Positive (n=5)

[9] McPartland JM, MacDonald C, Young M, Grant PS, Furkert DP, Glass M. Affinity and Efficacy Studies of Tetrahydrocannabinolic Acid A at Cannabinoid Receptor Types One and Two. Cannabis Cannabinoid Res. 2017;2(1):87-95.

Below administrative threshold (N1-N7)						
N1	NIDA	0.12	Negative (n=5)	Negative (n=5)	Negative (n=5)	Negative (n=5)
N2	NIDA	0.37	Negative (n=5)	Negative (n=5)	Negative (n=5)	Negative (n=5)
N3	Commercial hemp	<LOQ	Negative (n=5)	Negative (n=5)	Negative (n=5)	Negative (n=5)
N4	Commercial hemp	<LOQ	Negative (n=5)	Negative (n=5)	Negative (n=5)	Negative (n=5)
N5	Commercial hemp	<LOQ	Negative (n=5)	Negative (n=5)	Negative (n=5)	Negative (n=5)
N6	Commercial hemp	0.21	Negative (n=5)	Negative (n=5)	Negative (n=5)	Negative (n=5)
N7	Commercial hemp	0.21	Negative (n=5)	Negative (n=5)	Negative (n=5)	Negative (n=5)

Table 11. Summary of assay performance for individual and combined sites.

Site	**TP**	**TN**	**FP**	**FN**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
SHSU	35	35	0	0	100%	100%	100%	100%
HCIFS	30	35	0	5	86%	100%	100%	88%
HFSC	34	35	0	1	97%	100%	100%	97%
DPS	33	35	0	2	94%	100%	100%	95%
All Sites	**132**	**140**	**0**	**8**	**94%**	**100%**	**100%**	**95%**

9. Extract Stability

The stability of processed samples (*i.e.* extracts) was evaluated using *Cannabis sativa* extracts containing Δ9-THC above and below the administrative threshold. Processed samples (plant extracts) were extracted once and injected at least twice over five days following refrigerated storage in the dark. Mean decision-point ratios during five days of storage are summarized in Table 12.

Table 12. Processed sample stability over five days.

Site	Change in Decision-Point Ratio (relative to Day 1) over Five Days	CV over Five Days	Plant Matrices	Δ9-THC (% by Weight)
SHSU	-6% to 8% (n=4)	2.5-3.3%	P1, P2, P6, N3, N4	<LOQ to 3.9%
HCIFS	-8% to 10% (n=2)	5.8%-12.5%	P1, P2, P6, P7, N6	<LOQ to 10.10%
HFSC	-10% to 7% (n=2)	1.6-5.1%	P2, P4, P7, N3, N4	<LOQ to 6.7%
DPS	-10% to 7% (n=2)	8.3% - 12.5%	P1, P2, P6, N1, N2	0.12 - 10.10%

10. Decarboxylation

Conversion of Δ9-tetrahydrocannabinolic acid (THCA) to Δ9-THC in the GC inlet was estimated by comparison of peak areas following the injection of THCA and Δ9-THC (n=4). Lower rates of decarboxylation effectively increase the threshold for a positive result. Decarboxylation rates using an inlet temperature of 250°C are summarized in Table 13. Lower decarboxylation rates potentially increase the false negative rate (and negative predictive value) at the administrative threshold.

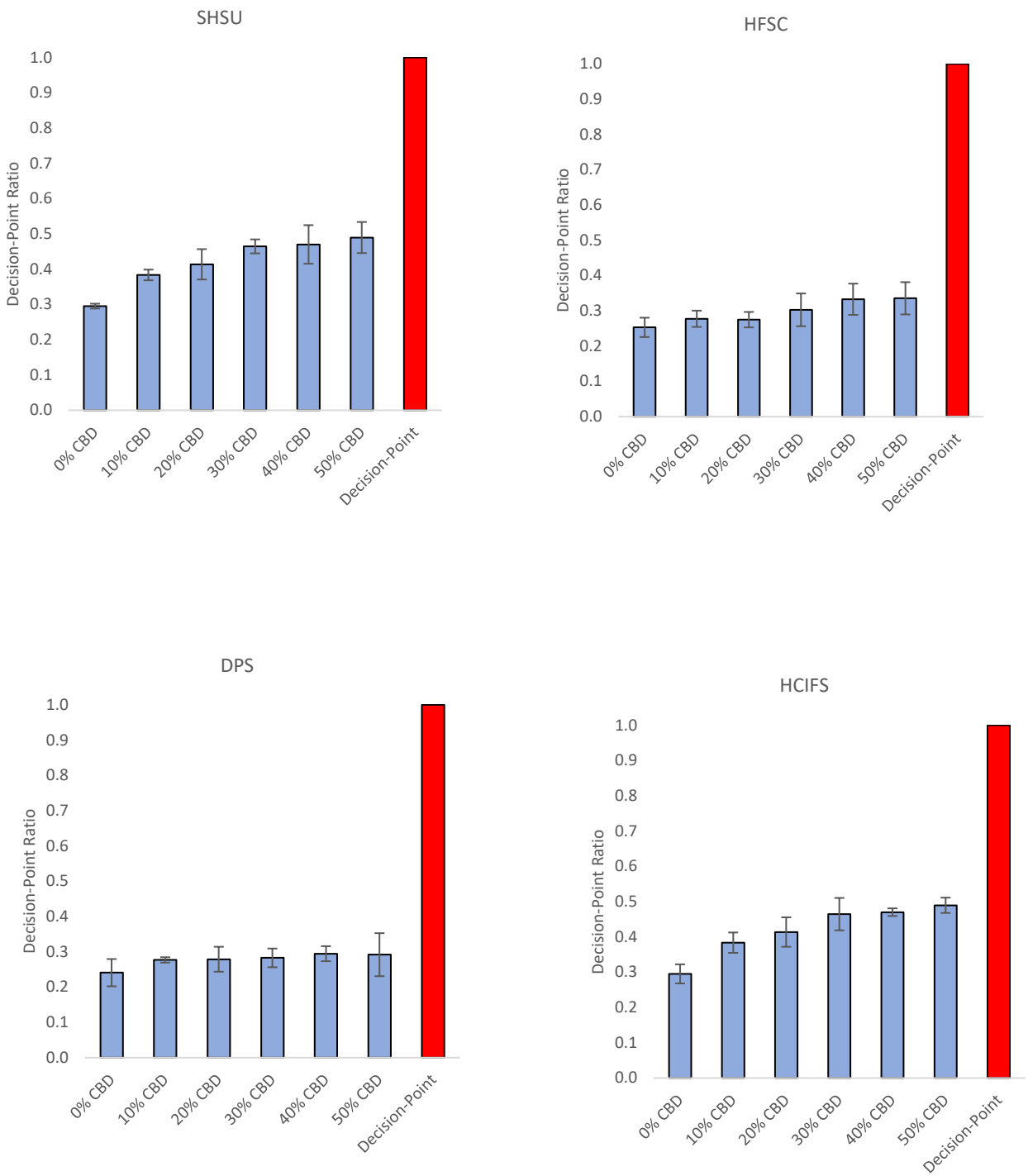
Table 13. Estimated rate of decarboxylation at an inlet temperature of 250°C.

Site	Decarboxylation Rate (n=4)
SHSU	62 ± 4
HCIFS	32 ± 7
HFSC	56 ± 22
DPS	42 ± 7

11. Cannabidiol Interference

The potential for CBD interference was evaluated. Negative control (IS only) and plant extracts (0.3% Δ9-THC) were fortified with the equivalent of 0, 10%, 20%, 30%, 40% and 50% CBD. A single injection of five independently prepared controls or extracts were analyzed. The decision point ratio was evaluated in the presence of 0-50% CBD by weight. No reportable Δ9-THC was detected in the negative control at concentrations up to 50% CBD. Decision-point ratios for the 0.3% Δ9-THC plant extract are shown in Figure 2.

Figure 2. Influence of CBD on the decision-point ratio with increasing CBD concentration in plant.



12. Dilution Integrity

Placebo extract was fortified with Δ9-THC to achieve concentrations of 0, 20, 30, 40 and 50% (by weight). Extracts were diluted with methanol post-extraction and decision-point ratios were evaluated to ensure acceptable results (> 1.0). Replicate placebo extracts were prepared (n=4) at each Δ9-THC concentration. The acceptability of results using five and ten-fold dilutions of plant extract are summarized in Table 14.

Table 14. Dilution integrity.

Site	Dilution Factor Evaluated	20% Δ9-THC	30% Δ9-THC	40% Δ9-THC	50% Δ9-THC
SHSU	10-fold	Acceptable	Acceptable	Acceptable	Acceptable
HCIFS	5-fold	Acceptable	Acceptable	Acceptable	Acceptable
HFSC	10-fold	Acceptable	Acceptable	Acceptable	Acceptable
DPS	5-fold	Acceptable	Acceptable	Acceptable	Acceptable

13. Measurement Uncertainty

Measurement uncertainty is not required for qualitative reporting purposes. However, measurement uncertainties were estimated using extracts of *Cannabis sativa* fortified with Δ9-THC at 0.3% by weight using a 95.45% confidence interval (k=2). A total of 30 *Cannabis sativa* extracts were utilized at each site and results are summarized in Table 15.

Table 15. Estimated uncertainty for *Cannabis sativa* plant material containing 0.3% Δ9-THC.

Site	Expanded Uncertainty for the Method	Expanded Uncertainty at 0.3% Δ9-THC in Plant Extract (Expressed in the appropriate units of measurement)
SHSU	12.2%	0.3 ± 0.04
HCIFS	21.8%	0.3 ± 0.07
HFSC	14.9%	0.3 ± 0.04
DPS	16.4%	0.3 ± 0.06