HOUSTON FORENSIC SCIENCE CENTER
COMPARATIVE AND ANALYTICAL DIVISION

VALIDATION OF AGILENT_7 GC/MS DECISION-POINT ASSAY FOR DELTA-9-THC IN PLANT SUBSTANCE

CAD Section: Seized Drugs
Type of Analysis: Qualitative Identification and Decision-Point Assay at a 1% Threshold for delta-9-THC by Gas Chromatography/Mass Spectrometry

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Purpose

The objective of this study is to validate a decision-point assay using the Agilent_7 Gas Chromatograph/ Mass Spectrometer (GC/MS) for the qualitative identification of unknown substances including but not limited to natural cannabinoids found in the plant species *Cannabis sativa L.* by mass spectral fragmentation patterns. The assay also uses an administratively determined threshold for delta-9-tetrahydrocannabinol (THC) at 1% as part of an analytical scheme for evaluating whether plant substance samples are marihuana.

To demonstrate that a method is fit for its intended purpose, mass fragmentation patterns should be reliable for multiple injections and deliver valid results when compared against library or reference searches. Additionally, a method must demonstrate selectivity. A selective method is one that provides sufficient baseline separation of compounds to allow for the identification of the analyte of interest. Therefore, retention times related to the mass spectral analysis of known cannabinoid standards will be used to demonstrate selectivity. Finally, the ability of the assay to reliably identify if the delta-9-THC concentration in plant substance is above or below the established threshold of 1% should be demonstrated through testing of plant samples with known concentrations.

Background

On June 10, 2019, the statutory definition of marihuana in the Texas Health and Safety Code Chapter 481.002.26 changed to exclude hemp as defined by Agriculture Code 121.001. Relevant portions of both statutes are cited below:

> "Marihuana means the plant *Cannabis sativa L.*, whether growing or not, the seeds of that plant, and every compound, manufacture, salt, derivative, mixture, or preparation of that plant or its seeds. The term does not include:
> (A) the resin extracted from a part of the plant or a compound, manufacture, salt, derivative, mixture, or preparation of the resin;
> (B) the mature stalks of the plant or fiber produced from the stalks;
> (C) oil or cake made from the seeds of the plant;
> (D) a compound, manufacture, salt, derivative, mixture, or preparation of the mature stalks, fiber, oil, or cake;
> (E) the sterilized seeds of the plant that are incapable of beginning germination; or
> (F) hemp, as that term is defined by Section 121.001, Agriculture Code."
>
> "Hemp means the plant *Cannabis sativa L.* and any part of that plant, including the seeds thereof and all derivatives, extracts, cannabinoids, isomers, acids, salts, and salts of isomers, whether growing or not, with a delta-9 tetrahydrocannabinol concentration of not more than 0.3 percent on a dry weight basis."

This change requires that laboratories demonstrate suspected marihuana samples not only contain delta-9-THC but also that its concentration is greater than 0.3% to distinguish marihuana from hemp.

The Seized Drugs section currently utilizes GC/MS analysis as a primary analytical technique for the identification of unknown substances including natural and synthetic cannabinoids submitted to the section. In addition to being used in the qualitative identification of substances, GC/MS may also be used to determine if the concentration of a substance is above (greater than) or below (less than) a specified amount. This specified amount may be referred to as a decision-point. The analytical

procedure for identification of a substance along with the determination of whether the concentration of that substance is above or below the decision-point is the decision-point assay.

The GC/MS method included in this decision-point assay utilized both the full scan and selected ion monitoring modes for data acquisition. Collectively, this is referred to as SIM-scan mode. Using the synchronous SIM-scan function, the instrument rapidly switches between the two modes allowing both sets of data to be collected. The full scan data produces a traditional mass spectrum which can be used for qualitative identification of substances in a sample. The SIM mode collects data for specified ions associated with substances of interest allowing for enhanced sensitivity and is used in determining if the amount of a substance is greater than or less than the decision-point.

This study describes the validation of the analytical procedure for extraction of cannabinoids including delta-9-THC from plant samples as well as analysis of these extracts using the Agilent_7 GC/MS instrumental method. Deuterated-delta-9-THC (THC-D3) was included as an internal standard in the assay to improve precision of the results.

Equipment:

The equipment and materials used in this study are as follows:

- Agilent 7890B Gas Chromatogram/5977A Mass Spectrometer System (Serial numbers: GC – CN15173178, MS – US1521L410) with 150 position auto-sampler, and auto-sampler injection tower. Agilent MS Masshunter software B07.06.2704
- Agilent DB-5-MS Column - 30m x 0.250mm x 0.25microm (5% Phenyl-methylpolysiloxane)
- BrandTech Scientific Organic Dispensette, Adjustable 0.5 - 5 mL
- 10-100µL Fisherbrand Elite pipette
- 100-1000µL Fisherbrand Elite pipette
- Mettler Model XP204 Analytical Balances
- Volumetric flasks (Class A)
- Glass culture tubes with caps (16x100mm)
- Autosampler vials with inserts and caps

GC/MS Method (CB_LONG_SIMSCAN) Parameters:

Initial Oven Temperature:	200°C hold for 0.0 min
First Ramp:	15°C /min to 235°C hold for 7.0 min
Second Ramp:	30°C/min to 290°C for 1.0 min
Front Inlet:	250°C
Carrier Gas:	Helium
Inlet Split Flow:	100:1 initial, 16:1 after 2 minutes
Column Flow:	1.5 mL/min (constant)
Linear Velocity:	47.523 cm/second
Syringe size:	5 µL
Injection Volume:	1 µL
Solvent rinse:	MeOH (A&B) ; 2 µL
Solvent Wash:	2A, 2B (Preinj) ; 10A, 10B (Postinj)
Viscosity delay:	2 seconds

Transfer Line:	280°C
MSD Source:	230°C
MSD Quad:	150°C
Tune Type:	Stune
Acquisition Mode	SIM/Scan
Scan Parameters	40.0 (low m/z) to 550 (high m/z)
SIM (THC)	314, 231, 271 (40 ms dwell)
SIM (THC-D3)	317, 234, 274 (40 ms dwell)
Solvent Delay:	2.0 minutes
Total Run Time:	12.167 minutes

Chemicals:

- 1 mg/mL delta-9-tetrahydrocannabinol certified reference material in methanol, Cayman Chemical # ISO60157
- 0.1 mg/mL delta-9-tetrahydrocannabinol-D3 certified reference material in methanol, Cayman Chemical # 19332
- Phytocannabinoid Mix 3 (1mg/ml each of cannabidiol, delta-9-tetrahydrocannabinol, and cannabinol) certified reference material in methanol, Cayman Chemical # 23251
- HFSC In-house verified standards
- OmniSolv HPLC grade Methanol (MeOH)

Preparation of solutions:

0.05 mg/mL delta-9-THC Standard Solution

Using a volumetric pipette, 500 µl of the delta-9-THC certified reference material was transferred to a 10 mL volumetric flask. The solution was brought to volume with MeOH. The resulting solution was stored in the freezer when not in use. This solution was equivalent to the 1% delta-9-THC in plant extract decision-point.

0.1 mg/mL delta-9-THC-D3 Internal Standard Solution (ISS)

The ISS consists of 0.1 mg/mL of delta-9-THC-D3 in MeOH which was purchased from Cayman Chemical and used directly as supplied. The solution was stored in the freezer when not in use.

0.05 mg/mL Secondary Standard Solution

Using a volumetric pipette, 500 µl of the Phytocannabinoid Mix 3 certified reference material was transferred to a 10 mL volumetric flask. The solution was brought to volume with MeOH. The resulting solution was stored in the freezer when not in use.

Positive Control (Decision-Point Control)

To prepare the positive (decision-point) control equal volumes of the 0.05 mg/mL delta-9-THC Standard Solution and the 0.1 mg/mL ISS were mixed. The solution was stored in the freezer when not in use.

Secondary Control

To prepare the secondary control equal volumes of the 0.05 mg/mL Secondary Standard Solution and the 0.1 mg/mL ISS were mixed. The solution was stored in the freezer when not in use.

Negative Control
To prepare the negative control equal volumes of MeOH and the 0.1 mg/mL ISS were mixed. The solution was stored in the freezer when not in use.

Plant Substance Extract
Using an analytical balance, 50 mg ± 0.5 mg of plant substance was weighed and transferred to a glass culture tube. Using the Organic Dispensette, two 5 mL aliquots of MeOH were added for a total volume of 10 mL. The mixture was vortexed for 10 seconds, allowed to stand for 5 minutes, and vortexed for an additional 10 seconds. Following this procedure, plant substance having a delta-9-THC concentration of 1% would result in an extract having a concentration of 0.05 mg/mL which is equivalent to the positive control (decision-point control).

For GC/MS analysis equal volumes of the plant extract and the 0.1 mg/mL ISS were mixed in an autosampler vial. The same ISS was used to prepare the extracts and the controls.

Scope of Assay Validation:
A qualification study documenting that an instrument is suitable for its intended purposes should be completed prior to the validation of an analytical method. The use of validated methods in a qualified instrument provides confidence in the reliability of separation, identification and categorization of analyzed samples. Refer to the Qualification Study for Agilent_7 GC/MS and Ancillary Equipment for instrumental performance specifications completed prior to this validation study.

The data used in this validation was compiled from numerous runs on multiple days by different analysts. The validation criteria used in this study were selectivity, detection limit, linearity, carryover, precision, decarboxylation, accuracy, processed sample stability, dilution integrity, and interferences by CBD. An uncertainty of measurement assessment was also conducted at the statutory level of 0.3% delta-9-THC and at the administrative decision-point level of 1%. Summary spreadsheets of the data collected as well as a solution preparation document are available in the Appendix. The raw data files are available electronically.

Selectivity:
Selectivity evaluates the ability of a method to separate analyte(s) in the presence of other commonly encountered substances. This criterion ensures that the method can identify the analyte(s) of interest. This assay is focused on identification of cannabinoids that occur naturally in *Cannabis sativa L.* (referred to simply as Cannabis), but more specifically the cannabinoid delta-9-THC. The first step in the validation study was to establish the selectivity of the instrumental method by using a variety of cannabinoids known to occur in Cannabis and a selected number of synthetic tetrahydrocannabinoids as well as phencyclidine (PCP is sometimes encountered in evidence submissions spiked onto marihuana samples). All of these substances were purchased standards verified in-house by GC/MS prior to use.

A mixture of the standards to be evaluated was prepared and used for the study. Intra-assay retention time stability was evaluated with 10 replicate injections of the 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). Data from the GC/MS method full scan mode was evaluated for this study.

The intra-assay (same day) average retention times for delta-9-THC, internal standard (delta-9-THC-D3), and other standards are shown in Table 1. A sample total ion chromatogram (TIC) is shown in Figure 1.

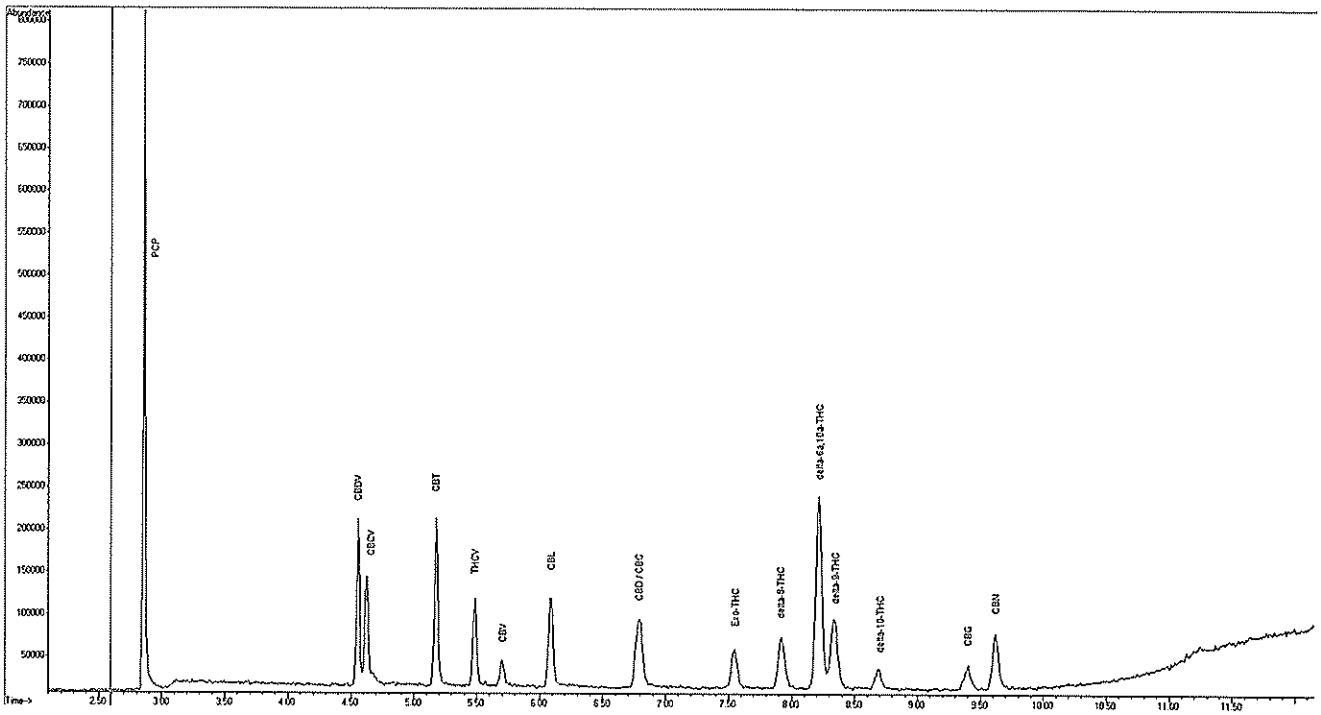
PCP is shown for reference. The method shows excellent selectivity for delta-9-THC from the other plant-based cannabinoids as well as exo-THC and delta-10-THC (both synthetic isomers of THC) with baseline separation and relative retention times (RRT) greater than 1% and 0.1 minutes. However, baseline separation of delta-9-THC from delta-6a,10a-THC which is also a synthetic isomer is not achieved even though the RRT is greater than 1%. This is not a concern as delta-6a,10a-THC is not expected to be present in plant samples analyzed with this assay. The two naturally occurring cannabinoids CBD and CBC are well separated from delta-9-THC but are not separated from each other using this method as seen by the single peak on the TIC. Identification of these two substances is possible however as CBD elutes first at the front end of the peak and CBC elutes second at the back end of the peak. By selecting data points at either end of the peak, mass spectra can be generated that provide acceptable identification of the two substances. Mass spectra for each of the standards were verified by comparison with the In-House library version 4 and found to be acceptable.

Retention time stability is demonstrated by the intra- and inter-assay data. The relative standard deviation (RSD) for the same day injections was 0.0% for delta-9-THC and ranged from 0.0 – 0.10% for the other cannabinoids. The RSD for the ten-day series of injections was 0.06% for delta-9-THC and ranged from 0.0 – 0.09% for the other cannabinoids. These values are well below an acceptance criteria of 1% and show excellent stability for this method.

Table 1. Selectivity – Average Retention Times from Intra-assay Data

	Absolute Retention Time (min)	Relative Retention Time (min)	Relative Retention Time (%)
Phencyclidine (PCP)	2.855	5.481	65.75%
Cannabidivarin (CBDV)	4.559	3.777	45.31%
Cannabichromevarin (CBCV)	4.626	3.710	44.51%
Cannabicitran (CBT)	5.177	3.159	37.90%
Tetrahydrocannabivarin (THCV)	5.486	2.850	34.19%
Cannabivarin (CBV)	5.703	2.633	31.59%
Cannabicyclol (CBL)	6.085	2.251	27.00%
Cannabidiol (CBD)	6.787	1.549	18.58%
Cannabichromene (CBC)	6.787	1.549	18.58%
Exo-tetrahydrocannabinol (Exo-THC)	7.547	0.789	9.46%
Delta-8-tetrahydrocannabinol	7.919	0.417	5.00%
Delta-6a,10a-tetrahydrocannabinol (delta-6a,10a-THC)	8.216	0.120	1.44%
Delta-9-tetrahydrocannabinol-D3 (delta-9-THC-D3)	8.282	0.054	0.65%
Delta-9-tetrahydrocannabinol (delta-9-THC)	**8.336**	**0.000**	**0.00%**
Delta 10-tetrahydrocannabinol (delta-10-THC)	8.687	0.351	4.21%
Cannabigerol (CBG)	9.398	1.062	12.74%
Cannabinol (CBN)	9.619	1.283	15.39%

Figure 1. Representative TIC



Linearity and Limit of Detection (LOD):
Linearity measures the relationship of an analyte's concentration and instrument response over a working range. A method can be considered linear when the concentration and response are directly proportional to one another. This linear relationship can by evaluated by calculating the correlation coefficient (r^2) which should be >0.99. The linearity of this assay was evaluated above and below the decision-point of 1% delta-9-THC which corresponds to a concentration of 0.05 mg/mL in a plant extract when prepared according to the assay procedure. The limit of detection (LOD) is the lowest concentration of an analyte that can be detected in the method.

The data for this study was evaluated using SIM mode. In this mode an ion from the mass spectrum of the compound of interest is selected to be the target ion and two other ions are selected to be qualifier ions. For delta-9-THC the target ion selected was 314 and the qualifier ions selected were 231 and 271. For the internal standard (delta-9-THC-D3) the target ion selected was 317 and the qualifier ions selected were 234 and 274 as these are the corresponding ions in the deuterated isomer. When a sample is injected, the GC/MS instrument generates a count of the number of these ions produced for a specified time. This ion count is referred to as abundance or peak area response. The ion count for the target ion is proportional to the amount of the compound present in the sample. The ion count for the qualifier ions is compared to the ion count for the target ion and must be within a specified percentage relative to the target ion for the data to be considered acceptable. For this assay the following acceptance criteria were established for delta-9-THC and delta-9-THC-D3: retention times should be within 1% and qualifier ion ratios should be within 20% of the expected values when compared to a positive (decision-point) control sample.

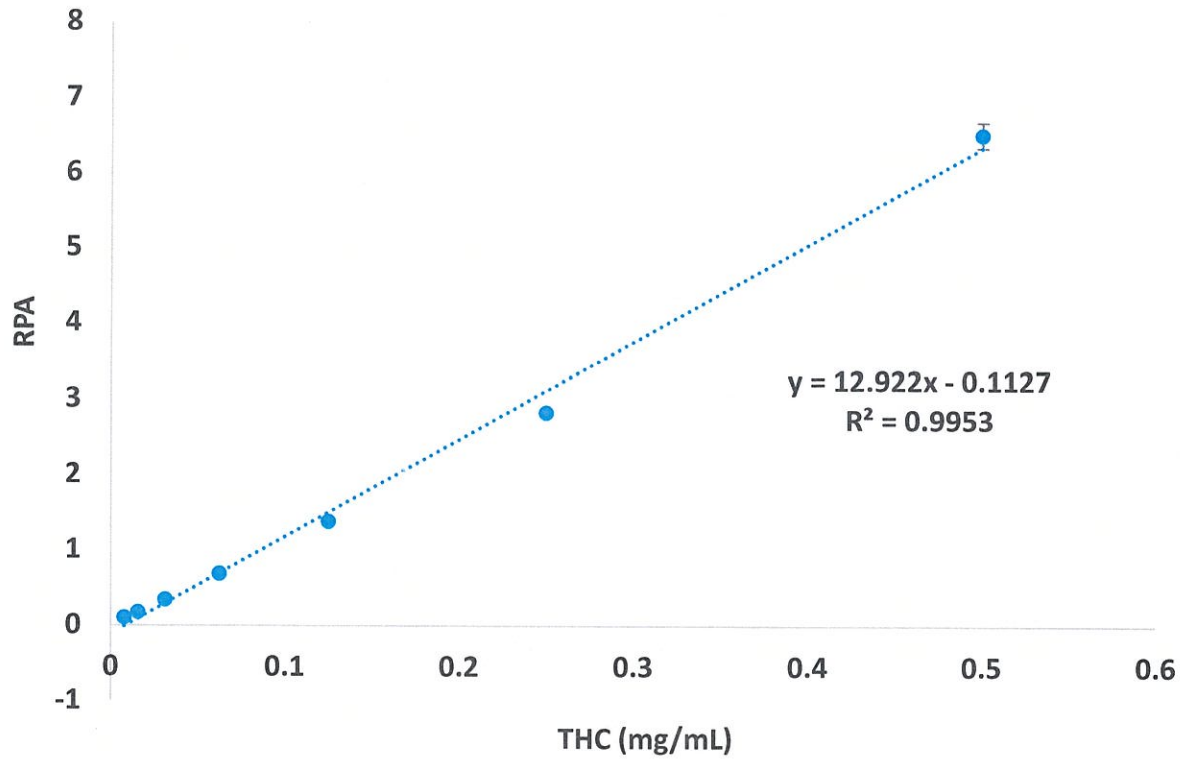
The linearity and LOD for the method were determined using serial dilutions of delta-9-THC certified reference material in methanol with 4 injections at each concentration. The series included the

following concentrations: 1.0, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156, 0.0078, 0.0039, 0.0019, and 0.0009 mg/mL delta-9-THC. Equal volumes of these solutions and 0.1 mg/mL ISS were mixed to prepare the final samples for injection.

The instrument responses were evaluated for each injection. Delta-9-THC was not detected in the 0.0009 or 0.0019 mg/mL solutions. The qualifier ion ratios were outside of the acceptable 20% range for all of the injections at the 0.0039 mg/mL solution. The lowest solution concentration to produce both detectable delta-9-THC and acceptance results was 0.0078 mg/mL, so this was determined to be the LOD for the assay method. This value corresponds to a plant extract concentration of 0.15% which is well below the decision-point value of 1%.

The relative peak area response (RPA, ratio of the abundance of delta-9-THC 314 ion and delta-9-THC-D3 317 ion) was determined for each injection and averaged to determine a final value of RPA for each concentration. These average RPA values were plotted against the corresponding concentration to determine the linear range. The lowest concentration value plotted was 0.0078 mg/mL which was the LOD. It was concluded that the plot is linear between 0.0078 mg/mL and 0.5 mg/mL as a correlation coefficient (r^2) of 0.995 was obtained for this range. This range corresponds to a plant extract concentration of 0.15% - 10%. It should be noted that while this linear range is well below and well above the decision-point value of 1%, this is not intended to be a limit on acceptability of samples that may extend above the 10% concentration range. As long as the acceptance criteria for sample data is met, the results should be considered acceptable. The linear plot calibration is shown in Figure 2.

Figure 2. Linearity Plot of Relative Peak Area vs. Concentration



Carryover

Carryover can be defined as the error induced in the analysis of a sample by contamination from the preceding one. Carryover was first assessed using two methanol blanks injected immediately after increasingly higher concentrations of delta-9-THC (1, 2, 3, and 4 mg/mL; equivalent to 20, 40, 60, and 80% delta-9-THC in plant extracts). The sequence of high delta-9-THC followed by methanol blanks was repeated five times. There was no detectable delta-9-THC identified in the methanol blanks throughout the study.

Carryover was also assessed by measuring the possible effect on a decision-point control. For this study a decision-point control (0.05 mg/mL delta-9-THC spiked with an equal amount of ISS) was injected to establish a known RPA (ratio of delta-9-THC/delta-9-THC-D3 response). Next a high concentration of delta-9-THC was injected followed by a methanol blank and then a reinjection of the decision-point control. This cycle of injections was repeated five times for delta-9-THC concentrations of 1, 2, 3, 4, and 4.5 mg/mL (equivalent to 20, 40, 60, 80, and 90% delta-9-THC in plant extracts). Carryover was considered to be absent if the post decision-point control was within 20% of the pre decision-point control which was the case throughout this study. Interestingly, two of the methanol blanks in the 4.0 mg/mL cycle did detect 314 ions associated with delta-9-THC; however, both of the qualifier ions (234 and 274) from the IS were outside of the ±20% range so the sample runs would be considered unacceptable. In fact, review of the data for both carryover studies showed that data at or above 3 mg/mL delta-9-THC (equivalent to 60% in plant extract) would be unacceptable as the qualifier ions for IS were consistently out of range.

These studies related to carryover demonstrate that precautions such as the syringe wash after analyte injection and the split values for the instrumental method are adequate in addressing the issue of carryover. It is recommended that methanol blanks be injected prior to and immediately following sample runs to indicate whether carryover from a previous run is present.

Precision (Repeatability, Reproducibility)

Precision measures the closeness of values from replicated analytical results to each other. The precision of the method was determined by analyzing replicate injections of prepared decision-point controls. Repeatability (intra-day precision) was evaluated by using ten independently prepared decision-point controls each injected once on the same day (n=10). The RPA (ratio of delta-9-THC/delta-9-THC-D3 response) was determined for each injection and an average value was calculated. The relative standard deviation (RSD) for this study was 3.7%.

Reproducibility (inter-day precision) was evaluated by using ten injections of a freshly prepared decision-point control each day over five days (n=50). The RPA was determined for each injection and an average value for the 50 injections was calculated. The RSD for this study was 3.5%.

The RSD values for both precision studies were under 10% which was determined to be acceptable.

Decarboxylation

In Cannabis, cannabinoids including delta-9-THC are naturally synthesized with a carboxyl function group and are designated by name as the acid version of the compound such as delta-9-tetrahydrocannabinolic acid (delta-9-THCA). As the plant dries, these compounds undergo decarboxylation to lose the carboxyl group and produce their neutral form, for example THCA becomes THC. This process can also be expedited by increases in temperature. When determining the amount of

THC isomers present in a sample, the acidic and neutral versions must be considered. For example, are separate values for THCA and THC concentration desired or is the combined concentration of THCA and THC (referred to as total THC) sufficient? While some techniques such as liquid chromatography can separate acidic and neutral cannabinoids without chemical derivatization, gas chromatography (GC) can facilitate total cannabinoid determinations due to the decarboxylation that occurs in the high temperature injection port. Since this assay uses GC/MS instrumentation, the acidic forms of cannabinoids present in plant extracts will be converted (at least partially) to the neutral forms and decision-point values will be for total delta-9-THC.

This study evaluated the efficiency of decarboxylation of delta-9-THCA into delta-9-THC in the Agilent_7 GC/MS injection port using the assay method. Solutions of delta-9-THCA and delta-9-THC were prepared at 0.05 mg/mL in methanol and mixed with equal volumes of 0.1 mg/mL ISS. Both solutions were then injected four times. The RPA was determined for each injection and an average value was calculated for delta-9-THCA and delta-9-THC. The average RPA for delta-9-THCA was 0.32 and the average RPA for delta-9-THC was 0.57. Comparison of these values shows that the conversion of delta-9-THCA into delta-9-THC is approximately 56%, however the RSD for the four injections of THCA was 22% which shows the wide variability that can be expected even from the same samples. The incomplete conversion of the acidic form of delta-9-THC using this method means that decision-point values for plant substance extracts will most likely be lower than the actual total delta-9-THC concentration. This should be seen as a limitation of this assay as some plant substance samples may be identified as having delta-9-THC concentrations below the 1% decision-point when the actual value is above.

Accuracy

This assay determines whether the amount of delta-9-THC in a sample of plant substance is above or below a decision-point threshold of 1% which is in effect a binary classification test where results fall into one of two groups or outcomes. For this type of test, accuracy measures the fraction of all instances that are correctly classified.

Accuracy of the assay was evaluated using fourteen previously characterized Cannabis plant samples, each extracted five times and analyzed by the GC/MS method (n=70). Seven of the plant samples contained delta-9-THC above the decision-point threshold (1%) and seven were below. Nine of the plant samples were provided by the National Institute on Drug Abuse (NIDA) and contained between 0.12 – 10.1% delta-9-THC. Five of the plant samples were purchased commercial hemp samples that all contained less than 1% delta-9-THC with some containing high concentrations of cannabidiol (CBD) or cannabigerol (CBG). Characterization reports for the fourteen samples are included in the Appendix.

Each sample extract was evaluated against a decision-point control at the 1% threshold by determining the decision-point ratio (DPR). The DPR is the ratio of RPA for the sample extract to RPA for the decision-point control and in effect normalizes the sample extract so that values will either be at, above, or below 1.0. For the purposes of this validation, "positive" indicates the DPR for an extract was at or above 1.0 and that the data satisfied the acceptance criteria (delta-9-THC and IS retention times within 1% and qualifier ion ratios within 20% of the expected values when compared to the decision-point control). "Negative" indicates a DPR below 1.0 and/or that the acceptance criteria were not met. The chemical composition and assay results are summarized in Table 2.

The data or results for binary classifications such as this assay can be broken down into four categories: true positives (TP) which are the correct positive classifications, true negatives (TN) which are the correct negative classifications, false positives (FP) which are the incorrect positive classifications, and false negatives (FN) which are the incorrect negative classifications. These categories can be used to measure the performance of the assay. Accuracy is determined by the formula:

$$\text{Accuracy} = (TP + TN)/(TP + TN + FP + FN)$$

Two other performance measures are Positive Predictive Value (PPV) which is the probability that the DPR will be positive for plant samples above the administrative threshold, and Negative Predictive Value (NPV) which is the probability that the DPR will be negative for plant samples below the administrative threshold. These values are determined by the formulas:

$$PPV = TP/(TP+FP) \qquad NPV = TN/(FN+TN)$$

Evaluation of the data in Table 2 yields the following category values: TP=34, TN=35, FP=0, and FN=1. This means that 69 out of the 70 samples returned the expected outcomes so the accuracy is 0.98 or 98%. None of the results were incorrectly classified as being above the 1% threshold (no false positives), and the PPV was 1.0 or 100%. The only incorrect classification was for one of the extracts for the NIDA sample which was characterized as containing 1.9% delta-9-THC but was classified as being below 1% by the assay (one false negative) which resulted in the NPV being 0.97 or 97%. A more detailed review of the characterization provided by NIDA for this sample showed that the amount of delta-9-THCA was >1%. It is possible that incomplete conversion of THCA to THC during analysis led to the misclassification of the sample as being below the 1% threshold. This was discussed as a limitation of the assay earlier.

Table 2. Accuracy – Summary of chemical composition and assay results

Plant Sample	Source	Total Δ9-THC	Total Δ8-THC	Total CBD	Total CBN	Total CBG	Result	
							Positive	Negative
P1	NIDA	1.9	ND	0.17	0.52	NK	N = 4	N = 1
P2	NIDA	3.9	ND	0.01	0.38	NK	N = 5	
P3	NIDA	8.0	ND	0.09	0.62	NK	N = 5	
P4	NIDA	6.7	ND	0.02	0.48	NK	N = 5	
P5	NIDA	10.1	ND	0.04	0.89	NK	N = 5	
P6	NIDA	2.4	0.01	3.7	0.24	NK	N = 5	
P7	NIDA	2.4	0.01	3.8	0.25	NK	N = 5	

N1	NIDA	0.12	0.01	3.3	0.03	NK		N = 5
N2	NIDA	0.37	0.02	9.2	0.05	NK		N = 5
N3	Commercial hemp	<LOQ	<LOQ	21.8	<LOQ	0.67		N = 5
N4	Commercial hemp	<LOQ	<LOQ	18.3	<LOQ	0.51		N = 5
N5	Commercial hemp	<LOQ	<LOQ	14.8	<LOQ	0.59		N = 5
N6	Commercial hemp	0.21	<LOQ	<LOQ	<LOQ	19.1		N = 5
N7	Commercial hemp	0.21	<LOQ	<LOQ	<LOQ	17.3		N = 5

LOQ = Limit of Quantitation; ND = Not determined; NK = Not known

Processed Sample Stability

The stability over time of plant extracts was evaluated by using five of the previously characterized Cannabis plant samples also used in the Accuracy study. The five samples used were P2, P4, P7, N3, and N4. These plant samples were extracted once and injected twice for five days (n=10). The extracts were stored in the refrigerator when not in use.

Each injection of sample extract was evaluated against a decision-point control run the same day and the decision-point ratio (DPR) was determined in the same manner as for the Accuracy study. This resulted in 10 values of DPR for each of the five plant extracts over the five-day period. The RSDs for the DPR values for each plant extract were calculated and found to be as follows: sample P2 = 5.1%, sample P4 = 2.5%, sample P7 = 2.5%, sample N3 = 1.6%, and sample N4 = 4.2%.

These values are well under 10% and demonstrate that plant extracts can be prepared and will be stable for later GC/MS analysis for at least 5 days when stored in the refrigerator.

Dilution Integrity

A dilution integrity study was performed to demonstrate that plant extracts with high concentrations of delta-9-THC (≥1 mg/mL equivalent to 20%) could be diluted by known volumes and would produce results that meet acceptability criteria and also give reliable accuracy results.

This study used a placebo plant sample obtained from NIDA. This placebo was a Cannabis plant sample from which all cannabinoids had been previously extracted. Methanol extracts of the placebo were fortified with delta-9-THC in amounts equivalent to 20, 30, 40, and 50% (1.0, 1.5, 2.0, and 2.5 mg/mL). These fortified extracts were then diluted 1 to 10 with methanol. Four diluted fortified extracts were prepared at each delta-9-THC concentration (n=4) and analyzed by the GC/MS assay method.

Each injection was evaluated against a decision-point control run the same day and the decision-point ratio (DPR) was determined in the same manner as for the Accuracy study. This resulted in 4 values of DPR at each of the fortification levels. All of the diluted fortified extracts produced DPR values >1.0 as expected and met the acceptance criteria for retention times and ion ratios. The RSDs for the DPR values for each fortification level were calculated and found to be as follows: 20% level = 3.0%, 30%

level = 1.6%, 40% level = 2.1%, and 50% level = 2.8%. These results show that when plant samples with a high concentration of delta-9-THC (as demonstrated by DPR values >1.0) do not produce results that meet acceptance criteria, dilution of the plant extract by a factor of 1 to 10 (10x) can be expected to give accurate decision-point results that also meet acceptance criteria.

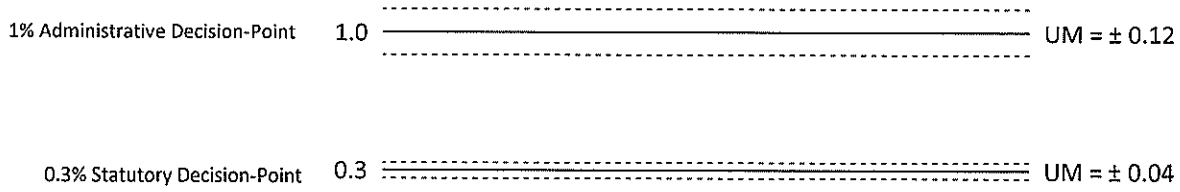
Uncertainty Assessment

In order to evaluate variability in the assay results an uncertainty of measurement was performed at the statutory level of 0.3% delta-9-THC and at the administrative decision-point threshold of 1%. Random (Type A) and systematic (Type B) uncertainty factors were considered and combined using a budget method to estimate the total expanded uncertainty at a confidence level of 95.45% (coverage factor of k=2).

To determine random uncertainty at the statutory level of 0.3%, a methanol extract of the NIDA placebo plant sample was fortified with 0.015 mg/mL delta-9-THC (equivalent to 0.3%). Six injections of the fortified extract were run each day for five days (n=30). The RPA was determined for each injection, and an average value for the 30 injections was calculated. The RSD for this study was 5.5%, and this value was used as the estimate of Type A uncertainty. To estimate the random uncertainty for the decision-point threshold of 1%, the reproducibility RSD value of 3.5% from the precision study was used. See the Uncertainty of Measurement Budget Tables in the Appendix for a summary of included systematic (Type B) factors and calculations.

The expanded uncertainty at the 0.3% statutory level was calculated to be ±0.04%. This demonstrates that plant samples with delta-9-THC concentrations at 0.3% will not be expected to reach the decision-point threshold of 1% due solely due to variability in the assay procedure. The expanded uncertainty at the 1% decision-point threshold was calculated to be ±0.12. This result indicates that it is possible for plant samples with delta-9-THC concentrations at 1% to yield results that are below the decision-point threshold due to variability in the assay procedure. A graphic representation of these results is shown in Figure 3.

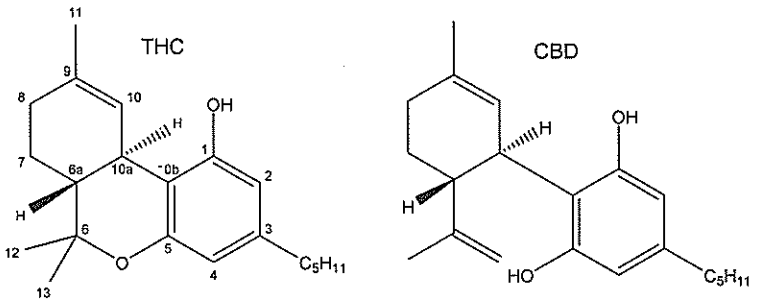
Figure 3. Graphic representation of expanded uncertainty



Interferences by CBD

Cannabidiol (CBD) is a naturally occurring cannabinoid present in varying amounts in Cannabis plant samples that can undergo cyclization to produce delta-9-THC. Structures of delta-9-THC and CBD are shown in Figure 4. While CBD is well separated from delta-9-THC in this method, the potential for interference exists due to the possible conversion of CBD to delta-9-THC in the GC/MS inlet.

Figure 4. Structures of delta-9-THC and CBD



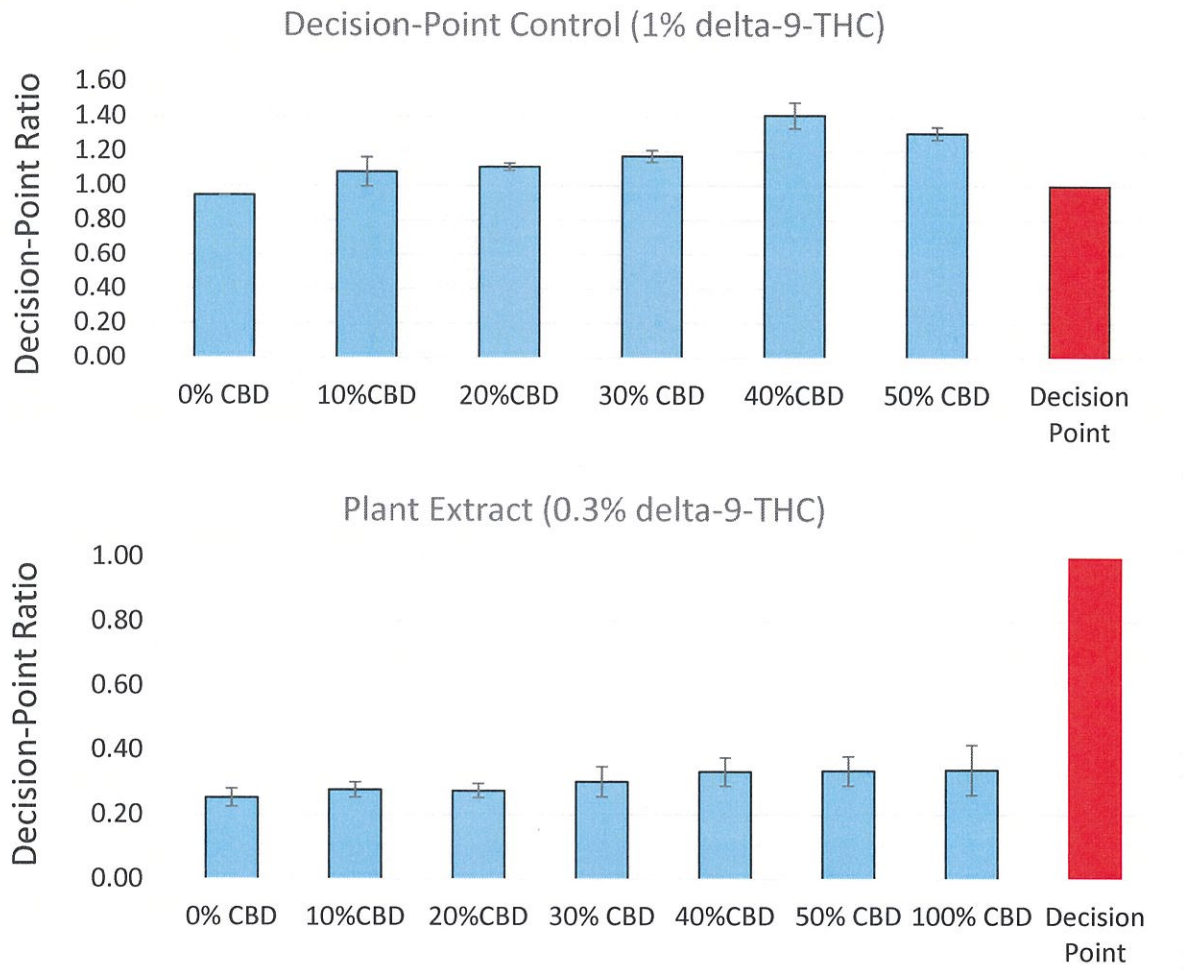
The potential for CBD interference was evaluated using the negative control (no delta-9-THC present), decision-point control (1% delta-9-THC present), and placebo plant extracts fortified with 0.3% delta-9-THC (the statutory level to distinguish marihuana and hemp). Negative control, decision-point control, and fortified placebo plant extracts were fortified with the equivalent of 0, 10%, 20%, 30%, 40% and 50% CBD. Out of interest, fortified placebo plant extracts were also fortified with the equivalent of 100% CBD to demonstrate what effect this would have on the results even though this scenario couldn't exist in an actual plant sample. A single injection of five independently prepared controls or extracts were analyzed. Each injection was evaluated against a decision-point control run the same day and decision-point ratios (DPR) were determined in the same manner as for the Accuracy study. In addition, the data from each injection was evaluated for acceptability of retention times and qualifier ion ratios when compared to the daily decision-point control sample.

None of the negative controls (no delta-9-THC) produced acceptable delta-9-THC results. All but one of the CBD fortified negative controls produced detectable 314 ions associated with delta-9-THC, but the ion ratios for the qualifier ions (231 and/or 271) were outside of the ± 20% range when compared with the daily decision-point control. All of the injections at the 0% CBD and one of the injections at the 10% CBD fortified level did not produce any 314 ions. This demonstrates that while CBD alone can produce ions associated with delta-9-THC, these results do not meet further criteria for acceptability and therefore would not result in identifiable delta-9-THC.

All but five of the decision-point control (1% delta-9-THC) injections produced acceptable results for the presence of delta-9-THC. The five unacceptable injections occurred at the 40% and 50% CBD levels and in these instances the 231 qualifier ion was outside of the ±20% range. Decision-point ratios for all injections at each of the CBD levels were averaged and this average is seen plotted versus the CBD levels in Figure 5. The individual and average DPR values at the 0% CBD level are all below 1.0 which would be a "negative" result even though the actual value is at the decision-point. This agrees with the results from the uncertainty study as the values fall within the calculated uncertainty range of ± 0.12 at the decision-point. The individual and average DPR values at the other levels of CBD (10 – 50%) are all above 1.0 which would be a "positive" result as expected. This demonstrates that samples containing delta-9-THC at the decision-point level of 1% and various concentrations of CBD will generally provide the expected result. However, high amounts of CBD can cause the results to be unacceptable when ion ratios are outside of the acceptable range.

All of the plant extract (0.3% delta-9-THC) injections produced detectable 314 ions associated with delta-9-THC as expected. However, with the exception of the 0% CBD level which yielded acceptable results, most of the results at the 10 – 100% CBD levels were unacceptable due to the 231 qualifier ion being outside of the ±20% range when compared with the daily decision-point control. In spite of this, decision-point ratios for all injections at each of the CBD levels were averaged and this average is seen plotted versus the CBD levels in Figure 5. The individual and average DPR values for all levels are well below 1.0 which would be a "negative" result as expected. In fact, the results agree well with the uncertainty study as the values for the 10 – 100% CBD levels fall within the calculated uncertainty range of ±0.04 at the 0.3% delta-9-THC level. The 0% CBD is just outside of the uncertainty range which may be the result of sample preparation variation. This demonstrates that samples containing various concentrations of CBD with delta-9-THC at or below the statutory limit of 0.3% would be expected to yield correct results.

Figure 5. Influence of CBD at the 1% delta-9-THC and 0.3% delta-9-THC levels



Conclusion

This completes the validation of a decision-point assay using the Agilent_7 GC/MS for the qualitative identification of unknown substances including but not limited to delta-9-THC as well as the determination as to whether the concentration of delta-9-THC present in *Cannabis sativa L.* plant samples is above or below an administrative threshold value (decision-point) of 1%. Factors included in this validation were selectivity, precision, linearity, limit of detection, and carryover. The testing showed that the GC/MS method has been optimized to qualitatively identify cannabinoids commonly associated with *Cannabis sativa L.* plant samples and separate them from the target compound delta-9-THC. The response of the instrument was also shown to be linear well above and well below the decision-point with a limit of detection of 0.0078 mg/mL (equivalent to 0.15% in plant using the described assay procedures). The %RSD for precision was under 5% which was determined to be acceptable. The potential for carryover from one sample to the next was addressed by including multiple syringe washes in the method and including dual blank runs between samples.

Another factor investigated was the decarboxylation of delta-9-THCA to delta-9-THC. The study showed that use of GC/MS instrumentation does facilitate this conversion, but since the conversion was found to be incomplete, the assay results for total delta-9-THC concentration in a plant sample could be lower than the actual amount especially in samples with a high level of delta-9-THCA. This is recognized as a limitation of the assay that could lead to false negative results.

The influence of high levels of cannabidiol (CBD), and the possible conversion of CBD into delta-9-THC through cyclization was investigated. The study evaluated the results of various levels of CBD without delta-9-THC and combined with 0.3% and 1.0% delta-9-THC. The only samples that did not give expected results were combinations of 40% and 50% CBD with 1.0% delta-9-THC. For these samples the acceptability criteria were not met so they would have led to false negative results.

The variability inherent in the assay itself was evaluated through uncertainty assessments at both the 0.3% delta-9-THC statutory threshold and at the administrative decision-point threshold of 1% delta-9-THC. The expanded uncertainty was calculated to be ±0.04% at the 0.3% level and ±0.12% at the 1% level. These results indicate that plant samples at the 0.3% level would not be expected to reach the 1% threshold, but that it is possible for plant samples at the 1% level to yield results that are below the decision-point threshold yielding false negative results.

The accuracy of the assay was evaluated using five extractions of fourteen previously characterized plant samples for a total of seventy extracts. Sixty-nine out of the seventy extracts gave the expected results for an accuracy of 0.98 or 98%. The one extract that did not give the expected result gave a value that was below the 1% decision-point instead of above 1%. This is possibly the result of incomplete conversion of delta-9-THCA into delta-9-THC. The positive predictive value for the assay was 100% and the negative predictive value was 97%. These results show that the assay gives excellent results when identifying a sample as being above the 1% level but does have limitations that can lead to false negative results where samples actually above the 1% level are incorrectly identified as being below 1%. One other limitation that can lead to variability in results is lack of homogeneity in the samples themselves. Since not all portions of plant material will contain the same concentration of cannabinoids, the results obtained may vary from sample to sample if steps are not taken to ensure a homogeneous representative sample is analyzed.

The stability of plant extracts was evaluated and showed that extracts that are kept in the refrigerator can be expected to give consistent results up to five days later. Dilution integrity was also evaluated and showed that plant extracts with high concentrations of delta-9-THC that do not meet acceptance criteria can be diluted by a factor of ten and will be expected to give accurate decision-point results that also meet acceptance criteria.

In conclusion, the data collected in this study has demonstrated that the decision-point assay method using Agilent_7 GC/MS is fit for its intended purpose of identifying unknown substances including but not limited to delta-9-THC as well as the determination as to whether the concentration of delta-9-THC present in *Cannabis sativa L.* plant samples is above or below an administrative threshold value (decision-point) of 1%. The assay results are to be incorporated as part of an overall analytical scheme for evaluating whether plant substance samples are marihuana. Although legislation sets a 0.3% statutory threshold to distinguish marihuana from hemp in *Cannabis sativa L.* samples, the administrative decision-point threshold was selected to encompass the variability in the assay procedure and to mitigate the risk of potential false positive results. This validation has shown that there may be instances where samples tested using this assay method may be reported as a false negative (i.e. *Cannabis sativa L.*), however the primary focus in the validation for this decision-point assay was to mitigate samples from being reported as false positives (i.e. marihuana).