Validation of a UPLC Method for Cannabinoids Concentration Quantification in Cannabis Flower

Objective

The purpose of this study is to validate a UPLC method using a Perkin Elmer Altus A-30 UPLC system in the Cannabis Testing Laboratory Branch. This method is used to quantify nine cannabinoids in cannabis flower sample. The nine cannabinoids of interest are CBDA, THCV, CBD, CBG, CBN, THCA, Δ9-THC, Δ8-THC and CBC.

Study Design

This study will follow the FDA's Guidelines for the Validation of Chemical Methods for the FDA FVM Program, 3rd Edition at the level three: multi-laboratory validation.

Nine individual cannabinoid standards were mixed to prepare the mixed cannabinoids standards at concentrations ranging from 0.5 ppm to 100 ppm. A seven-point standard calibration curve was generated for quantitation of the samples. Three samples were weighed out and analyzed from the ground cannabis flower sample for day 1 to evaluate the precision of the method. Another two samples were weighed out and analyzed on day 2, day 3 and day 4, respectively, to evaluate the reproducibility of the method. ACN:Methanol 80:20 was used as reagent blank. Cellulose powder was used as method blank. A mixture of the nine cannabinoids standards was also spiked into blank matrix samples (cellulose powder) as well as the reagent blank. Recoveries of the cannabinoids were calculated based on these spiked samples. Robustness of the method is evaluated by altering injection volume from 2ul to 3 ul and testing on another matrix, hemp, on day 4. Measurement uncertainty will be calculate using recovery of the matrix spike samples from the 4 different runs.

Materials and Equipment:

Cannabinoids Standards:

1. Cannabidiolic Acid (CBDA), 1.0 mg/ml, Cayman, Lot: 0587881
2. Tetrahydrocannabivarin (THCV), 1.0 mg/ml, Cayman, Lot: 0606999
3. Cannabidiol (CBD), 1.0 mg/ml, Cayman, Lot: 0586575
4. Cannabigerol (CBG), 1.0 mg/ml, Cayman, Lot: 0567652
5. Cannabinol (CBN), 1.0 mg/ml, Cayman, Lot: 0584229
6. Delta9-Tetrahydrocannabinol (Dleta9-THC), 1.0 mg/ml, Cayman, Lot: 0612973
7. Delta8-Tetrahydrocannabinol (Dleta8-THC), Cerilliant, Lot: FE12271903
8. Tetrahydrocannabinolic Acid (THCA), 1.0 mg/ml, Cayman, Lot: 0626035
9. Cannabichromene (CBC), 1.0 mg/ml, Cayman, Lot: 0586327
10. Cannabidlolic Acid (CBDA), 1.0 mg/ml, Cerilliant, Lot: FE02202007
11. Tetrahydrocannabivarin (THCV), 1.0 mg/ml, Cerilliant, Lot: FE10111901
12. Cannabidiol (CBD), 1.0 mg/ml, Cerilliant, Lot: FE10071912
13. Cannabigerol (CBG), 1.0 mg/ml, Cerilliant, Lot: FN03072001
14. Cannabinol (CBN), 1.0 mg/ml, Cerilliant, Lot: FE11211801
15. Delta9-Tetrahydrocannabinol (Dleta9-THC), 1.0 mg/ml, Cerilliant, Lot: FE02072001
16. Delta8-Tetrahydrocannabinol (Dleta8-THC), 1.0 mg/ml, Cerilliant, Lot: FE04282108

17. Tetrahydrocannabinolic Acid (THCA), 1.0 mg/ml, Cerilliant, Lot: FE11102003
18. Cannabichromene (CBC), 1.0 mg/ml, Cerilliant, Lot: FE06152005

Reagents:

1. Water, LC-MS grade
2. Methanol, LC-MS grade
3. Acetonitrile, LC-MS grade
4. Formic Acid, LC-MS grade

Cannabis flower sample (19-01597-CE), ground

Equipment:

1. Analytical balance Mettler Toledo XPE204
2. Disposable glass Pasteur pipette
3. Pipettes and pipet tips (20ul, 100ul, 1ml)
4. Conical polypropylene centrifuge tubes, 50ml
5. Centrifuge (capable of 4000 rpm)
6. Sonicator
7. HPLC vials, amber
8. HPLC caps
9. UPLC, PerkinElmer Altus A-30
10. Column: Waters Cortex C18 2.1 x 100mm, 1.6um
11. Disposable syringes with Luer-Lok tips, 5ml
12. Syringe filter disk, 0.2um PTFE
13. HPLC solvent bottles, 1L
14. Vortex mixer
15. Griffin glass beakers
16. Graduated cylinder

Methods:

LC parameters

1. Column: Restek Raptor ARC-18 2.1 x 150mm, 2.7um
2. Mobile phase A: Water with 0.05% formic acid
   Mobile phase B: Acetonitrile with 0.05% formic acid
3. Gradient Program:

Time (min)	Flow rate (mL/min)	% Mobile Phase A	% Mobile Phase B
0	0.4	25	75
7.00	0.4	25	75
7.01	0.4	0	100
9.00	0.4	0	100
9.01	0.4	25	75
12.00	0.4	25	75

4. Flow Rate: 0.4 mL/min
5. Run time: total 12.00 min: 7.00 min + 2 min washing period + 3 min column re-equilibration
6. Column Temperature: 35°C
7. Autosampler Temperature: 15°C
8. Injection Volume: 2 ul (day 1 to day 3) or 3 ul (day 4)

PDA detector

1. Spectrum data range: 210 - 400 nm
2. Wavelength for detection:

Compound	Wavelength for detection
CBDA	220 nm
CBG	220 nm
CBD	220 nm
THCV	220 nm
CBN	220 nm
Delta9-THC	220 nm
Delta8-THC	220nm
CBC	220nm
THCA	220 nm

Day 0

1. Prepare nine-cannabinoids-mix standards from the first source at concentration of 0.5, 2, 5, 10, 20, 50 and 100 ppm
    - Prepare 100 ppm first source (A) and 10 ppm first source (B) nine-cannabinoids working standards from the 1mg/ml cannabinoids stock standards. Store in freezer under -20 °C before use.
    - Prepare 20, 50 and 100 ppm calibration standards by appropriate dilution of the 100 ppm cannabinoids mix working standards (A) using acetonitrile/methanol (80:20) as diluent.
    - Prepare 0.5, 2, 5 and 10 ppm calibration standards by appropriate dilution of the 10 ppm cannabinoids mix working standards (B) using acetonitrile/ methanol (80:20) as diluent
2. Prepare working standards from the second source at concentration of 100 and 10 ppm.
    - Prepare 100 ppm second source (C) and 10 ppm second source (D), nine-cannabinoids working standards from the 1mg/ml cannabinoids stock standards. Store in freezer under -20 °C before use.
3. Prepare mobile phase A: Water with 0.05% formic acid; B: Acetonitrile with 0.05% formic acid. To prepare mobile phase A, add 0.5 ml formic acid to 1 L water. To prepare mobile phase B, add 0.5 ml formic acid to 1 L Acetonitrile.
4. Prepare method blank samples. Weigh 200 mg cellulose powder into 50ml centrifuge tube. Record the weight of the sample. Repeat this procedure to obtain 5 replicate samples (total 5 samples, 2 for day 1, 1 for day 2, 1 for day 3 and 1 for day 4).

5. Prepare cannabis flower and hemp samples. Weigh 200 mg ground flower samples into a 50 ml centrifuge tube. Record the weight of the sample. Repeat this procedure to obtain 9 replicate samples (total 9 samples, 3 for day 1, 2 for day 2, 2 for day 3 and 2 for day 4). Weigh 200 mg ground hemp samples into a 50 ml centrifuge tube. Record the weight of the sample. Repeat this procedure to obtain 3 replicate samples for day 4. Sample extraction and dilution is performed on the day of analysis: Add 40ml of 80:20 ACN:MeOH to each tubes with sample and vortex for 30 seconds to mix. Extract in a sonicating bath with ice for 30 minutes. Centrifuge at 3900 rpm for 15 minutes. Take the top layer liquid and filter each sample through a 0.2 um PTFE filter into an HPLC vial. Then dilute each sample 5 times with 80:20 ACN:MeOH.
6. Prepare matrix spiked samples at 3 spike levels
   Matrix Spike sample 1 (MS1): Weigh 200mg cellulose powder into 50ml centrifuge tube. Record the weight of the sample. Add 2 ml 100 ppm 9-mix standards to the tube.
   Matrix Spike sample 2 (MS2): Weigh 200mg cellulose powder into 50ml centrifuge tube. Record the weight of the sample. Add 1.4 ml 100 ppm 9-mix standards to the tube.
   Matrix Spike sample 3 (MS3): Weigh 200mg cellulose powder into 50ml centrifuge tube. Record the weight of the sample. Add 1 ml 100 ppm 9-mix standards to the tube.
   Extract both spike samples in a sonicating bath for 30 minutes. Centrifuge at 3900 rpm for 15 minutes. Take the top layer liquid and filter each sample through a 0.2 um PTFE filter into an HPLC vial.
   On day 2, day 3 and day 4, prepare a matrix spike sample following the same procedure as spike sample 3 of day 1, respectively.
7. Prepare method standard sample.
   Add 2 ml 100ppm 9-mix standards to the tube with 40ml 80:20 ACN:MeOH. Vortex for 30 seconds to mix.
   Extract method standard samples in a sonicating bath for 30 minutes. Centrifuge at 3900 rpm for 15 minutes. Filter each sample through a 0.2 um PTFE filter into an HPLC vial.
8. Prepare post-dilution spike sample
   Add the last step of sample dilution, add 25 ul sample extract and 200 ul 100 ppm 9-mix standards, then add 275 ul 80:20 ACN:MeOH. This is spiking 40 ppm of each cannabinoids to 20x dilution of the extracted flower sample.

Day 1

1. Extract and dilute 1 reagent blank, 2 method blanks, 1 method standard, 2 matrix spikes (3 levels in cellulose powder), 3 replicates of flower sample and 1 post dilution spike in flower sample.
2. Equilibrate the UPLC system with the mobile phases. Set injection volume at 2 ul.
3. Inject the standards used to generate the seven-point calibration curve and ICV sample using a set of second-source cannabinoids standards.
4. Inject the samples, including the 1 reagent blank, 2 method blanks, 1 method standard, 3 matrix spikes (3 levels in cellulose powder), 3 replicates of flower sample and 1 post dilution spike in flower sample.
5. After every 10 injections, re-inject a check standard using one of the calibration standards and a solvent blank (80:20 ACN: MeOH) for quality control purposes.
6. At the end of the run, re-inject a check standard using one of the calibration standards and a blank for quality control purposes

Day2

1. Extract and dilute 1 reagent blank, 1 method blanks, 1 matrix spike sample (1 level in cellulose powder), 2 replicates of flower sample and 1 post dilution spike in flower sample.
2. Equilibrate the UPLC system with the mobile phases. Set injection volume at 2 ul.
3. Inject the standards used to generate the seven-point calibration curve and ICV sample using a set of second-source cannabinoids standards.
4. Inject the samples, including the 1 reagent blank, 1 method blanks, 1 matrix spike sample (1 level in cellulose powder), 2 replicates of flower sample and 1 post dilution spike in flower sample.
5. After every 10 injections, re-inject a check standard using one of the calibration standards and a solvent blank (80:20 ACN: MeOH) for quality control purposes.
6. At the end of the run, re-inject a check standard using one of the calibration standards and a blank for quality control purposes.

Day3

1. Extract and dilute 1 reagent blank, 1 method blanks, 1 matrix spike sample (1 level in cellulose powder), 2 replicates of flower sample, and 1 post dilution spike in flower sample.
2. Equilibrate the UPLC system with the mobile phases. Set injection volume at 2 ul.
3. Inject the standards used to generate the seven-point calibration curve and ICV sample using a set of second-source cannabinoids standards.
4. Inject the samples, including the 1 reagent blank, 1 method blanks, 1 matrix spike sample (1 level in cellulose powder), 2 replicates of flower sample and 1 post dilution spike in flower sample.
5. After every <10 injections, re-inject a check standard using one of the calibration standards and a solvent blank (80:20 ACN: MeOH) for quality control purposes.
6. At the end of the run, re-inject a check standard using one of the calibration standards and a blank for quality control purposes.

Day 4 – alter injection volume, test on another matrix (hemp)

1. Extract and dilute 1 reagent blank, 1 method blanks, 1 matrix spike sample (1 level in cellulose powder), 2 replicates of flower sample, 1 post dilution spike in flower sample and 3 replicates of hemp sample.
2. Equilibrate the UPLC system with the mobile phases. Set injection volume at 3 ul.
3. Inject the standards used to generate the seven-point calibration curve and ICV sample using a set of second-source cannabinoids standards.
4. Inject the samples, including the 1 reagent blank, 1 method blanks, 1 matrix spike sample (1 level in cellulose powder), 2 replicates of flower sample, 3 replicates of hemp sample and 1 post dilution spike in flower sample.
5. After every <10 injections, re-inject a check standard using one of the calibration standards and a solvent blank (80:20 ACN: MeOH) for quality control purposes.
6. At the end of the run, re-inject a check standard using one of the calibration standards and a blank for quality control purposes

Acceptance Criteria

Calibration curves should have correlation coefficient >0.99. All the calibration check standards should be within 80-120% recovery. Matrix Spikes and post dilution spikes should be within 70-130% recovery. Method standard should be within 80-120% recovery.

Results and Discussion:

Calibration curve and range: 7 data points (not including 0) for all analytes ranges from 0.5 ppm - 100 ppm, using linear regression with 1/x weighting, R^2 value for all analytes are greater than 0.99.

Accuracy

Accuracy is assessed by the recovery of the QC samples below:

ICV: Initial calibration verification, second source standards run after calibration curve, recovery within 80-120%, please see attached QC reports for day 1 to day 4

CCV: Continuing calibration verification run after every <10 samples, recovery within 80-120%, please see attached QC reports for day 1 to day 5

Method standard recovery: using Cannabinoids Standards, 80-120%

Recovery% = Found/Amount Spiked X 100

Table 1: Recovery of Method Standard

	Recovery								
Method Std	CBDA	THCV	CBD	CBG	CBN	Δ9-THC	Δ8-THC	THCA	CBC
Found (mg)	0.1924	0.1952	0.1856	0.1850	0.1818	0.1914	0.1917	0.1810	0.1935
Amount Spiked (mg)	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000
Recovery	96%	98%	93%	92%	91%	96%	96%	91%	97%

Matrix spikes recovery: 70-130 % at two different spike levels using Cannabinoids Standards

Recovery% = Found/Amount Spiked X 100

Table 2: Recovery of Matrix Spike

	Matrix Spike								
Level 1	CBDA	THCV	CBD	CBG	CBN	Δ9-THC	Δ8-THC	THCA	CBC
Found (mg/g)	0.9629	0.9471	0.9268	0.9268	0.9289	0.9366	0.9392	0.9469	0.9303
Amount Spiked (mg/g)	0.9872	0.9872	0.9872	0.9872	0.9872	0.9872	0.9872	0.9872	0.9872
Recovery	98%	96%	94%	94%	94%	95%	95%	96%	94%
Level 2	CBDA	THCV	CBD	CBG	CBN	Δ9-THC	Δ8-THC	THCA	CBC
Found (mg/g)	0.7105	0.6982	0.6543	0.6577	0.6557	0.6890	0.6958	0.6782	0.6966
Amount Spiked (mg/g)	0.6976	0.6976	0.6976	0.6976	0.6976	0.6976	0.6976	0.6976	0.6976
Recovery	102%	100%	94%	94%	94%	99%	100%	97%	100%
Level 3	CBDA	THCV	CBD	CBG	CBN	Δ9-THC	Δ8-THC	THCA	CBC
Found (mg/g)	0.5000	0.4975	0.4909	0.4915	0.4899	0.4879	0.4903	0.4901	0.4849
Amount Spiked (mg/g)	0.4973	0.4973	0.4973	0.4973	0.4973	0.4973	0.4973	0.4973	0.4973
Recovery	101%	100%	99%	99%	99%	98%	99%	99%	98%

Post dilution spike recovery: 70-130% using Cannabinoids Standards

Recovery% = (Spiked – Unspiked/2)/Amount Spiked X 100

Table 3: Recovery of Post-dilution Spike

	Post-dilution Spike in 20x Sample Extract								
	CBDA	THCV	CBD	CBG	CBN	Δ9-THCe	Δ8-THCe	THCA	CBC
Unspiked 10x (mg/L)	0.00	0.00	0.00	0.91	1.91	28.74	1.62e	48.84	0.44
Spiked 20x (mg/L)	41.53	41.80	39.34	39.60	40.34	56.46	42.57e	67.46	41.82
Amount Spiked (mg/L)	40.00	40.00	40.00	40.00	40.00	40.00	40.00e	40.00	40.00
Recovery	104%	104%	98%	98%	98%	105%	104%e	108%	104%

Cannabinoids Concentration in flower samples:

All nine cannabinoids are identified in the cannabis flower sample. CBD is below the LOD. All raw data and calculation can be viewed in the attached spreadsheet.

Table 4: Concentration of nine cannabinoids in sample on day 1 (mg/g)

	Cannabinoids Concentration in Sample (mg/g)								
	CBDA	THCV	CBD	CBG	CBN	Δ9-THCe	Δ8-THCe	THCA	CBC
F1-1	0.487	0.361	ND	2.132	4.092	60.383	3.729e	104.339	0.984
F1-2	0.468	0.349	ND	2.025	3.819	57.288	3.648e	99.213	0.931
F1-3	0.463	0.353	ND	2.042	3.825	57.082	3.444e	97.003	0.919
Avg	0.472	0.354	ND	2.066	3.912	58.251	3.607e	100.185	0.945

Intra-Day Precision

Precision is evaluated by calculating the RSD% of the 3 replicate flower samples ran on the day 1, RSD% within 20%. As shown in Table 5, precision criterion has been met for all nine cannabinoids except for CBD, which was below LOD. All raw data and calculation can be viewed in the attached spreadsheet.

Table 5: RSD of concentration of nine cannabinoids in sample measured in day 1

	CBDA	THCV	CBD	CBG	CBN	Δ9-THC	Δ8-THCe	THCA	CBC
RSD	2.7%	1.8%	NA	2.8%	4.0%	3.2%	4.1%	3.8%	3.7%

Sensitivity

Sensitivity is the smallest amount of analytes in a sample that can be accurately measured the method. It is shown by analyzing blanks and lowest level matrix spike samples (0.1 mg/g) – LOD samples. LOD samples were prepared by Spike 20 ug of cannabinoids to blank matrix (cellulose powder), go through all sample prep procedures, this is equivalent to 0.1 mg/g in flower sample and 0.5 ppm in vial (lowest calibration point), prepare 7 sample replicates separately and run them in one sequence, calculate the LOD from the standard deviation (LOD = t x S, where t=3.14 for 7 replicates at 99% confidence level).

LOQ = 3 x LOD, should be in calibration curve and 1.0 mg/g or lower for all cannabinoids analyzed and reported.

Reagent Blank and Method blank

ACN:MeOH 80:20 was used as the reagent blank. Cellulose powder was used as the method blank. No cannabinoids peaks were identified in those blank sample.

LOD and LOQ:

LOD in sample = LOD x 0.04/0.2

LOQ in sample = 3 x LOD in sample

Table 6: Calculated LOD and LOQ

	CBDA	THCV	CBD	CBG	CBN	Δ9-THC	Δ8-THC	THCA	CBC
LOD-1 (mg/L)	0.571	0.545	0.527	0.523	0.522	0.541	0.491	0.476	0.51
LOD-2 (mg/L)	0.549	0.538	0.519	0.517	0.526	0.487	0.489	0.428	0.511
LOD-3 (mg/L)	0.599	0.535	0.503	0.508	0.513	0.497	0.492	0.488	0.528
LOD-4 (mg/L)	0.604	0.539	0.521	0.512	0.523	0.534	0.536	0.49	0.569
LOD-5 (mg/L)	0.587	0.547	0.504	0.521	0.524	0.533	0.516	0.467	0.515
LOD-6 (mg/L)	0.535	0.514	0.528	0.521	0.521	0.531	0.499	0.471	0.529
LOD-7 (mg/L)	0.542	0.525	0.515	0.526	0.523	0.56	0.565	0.474	0.482
Standard deviation (5)	0.028	0.012	0.010	0.006	0.004	0.025	0.029	0.021	0.026
LOD (mg/L)	0.088	0.036	0.032	0.020	0.013	0.080	0.090	0.065	0.083
LOQ (mg/L)	0.265	0.109	0.095	0.060	0.039	0.240	0.270	0.194	0.249
LOD in sample (mg/g)	0.018	0.007	0.006	0.004	0.003	0.016	0.018	0.013	0.017
LOQ in sample (mg/g)	0.053	0.022	0.019	0.012	0.008	0.048	0.054	0.039	0.050

However, pursuant to 4 CCR 15713(c)(1)(C)(iii), the laboratory shall ensure that the LOQ be within the range of the calibration curve. The calculated LOQs for CBDA, THCV, CBD, CBG, CBN, Δ9-THC, Δ8-THC, THCA, and CBC are not within the quantifiable range of the calibration curve, so they are raised to the lowest calibration point, which is 0.5 mg/L. Table 7 demonstrates the final LOQs.

Table 7: Final LOQs

	CBDA	THCV	CBD	CBG	CBN	Δ9-THC	Δ8-THC	THCA	CBC
LOQ (mg/L)	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500	0.500
LOQ in sample (mg/g)	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100	0.100

Reproducibility

Reproducibility is assessed by analyzing cannabis sample replicates on 3 different days to compare results (sample replicates are prepared separately). RSD% of the results for the flower samples in 3 days shall be within 20%. The average cannabinoids concentrations of each day are calculated. The RSD of cannabinoids concentrations are calculated for the day **1** to day 3. As shown in Table 8, the reproducibility criterion has been met.

Table 8: Cannabinoids Concentration for samples analyzed on 3 different days

	Cannabinoids Concentration in Sample (mg/g)								
	CBDA	THCV	CBD	CBG	CBN	Δ9-THC	Δ8-THC	THCA	CBC
Day1	0.472	0.354	ND	2.066	3.912	58.251	3.607	100.185	0.945
Day2	0.465	0.345	ND	2.003	3.864	56.662	3.575	97.967	0.938
Day3	0.453	0.340	ND	1.978	3.849	55.343	3.596	97.946	0.902
RSD	2.09%	2.04%	NA	2.25%	0.85%	2.57%	0.45%	1.30%	2.46%

Note: The concentration of each cannabinoid is the average of the samples analyzed in each day.

Precision and Reproducibility Calculated using Matrix Spikes

The precision and reproducibility are also evaluated by calculating the RSD% of the 4 matrix spikes, at 2.5 ppm in the vials, ran on the day **1**, 2, 3 and 4, RSD% within 20%. As shown in Table 9, precision and reproducibility criterion has been met for all nine cannabinoids. All raw data and calculations can be viewed in the attached spreadsheet.

Table 9: RSD of concentration of nine cannabinoids in matrix spikes at 2.5 ppm in the vials in day 1, 2, 3 and 4.

	CBDA	THCV	CBD	CBG	CBN	Δ9-THC	Δ8-THC	THCA	CBC
RSD	1.8%	0.9%	1.7%	1.6%	2.2%	1.4%	1.3%	2.8%	1.8%

Robustness

Robustness is evaluated by **1.** altering injection volume (2 ul vs 3 ul) and 2. testing on another matrix, hemp on day 4.

Results in Table 10 show that when applying a different injection volume, the method is still robust.

Table 10: Comparison for using different injection volume

	Cannabinoids Concentration in Sample (mg/g)								
	CBDA	THCV	CBD	CBG	CBN	Δ9-THC	Δ8-THC	THCA	CBC
Day 1-3*	0.465	0.348	ND	2.023	3.881	56.966	3.595	98.912	0.930
Day 4	0.477	0.354	ND	2.059	3.999	57.054	3.547	101.884	0.938
RPD	2.51%	1.75%	NA	1.77%	3.00%	0.15%	1.34%	2.96%	0.86%

Day1-3*: Average of all samples from day **1** to day 3.

Results in Table 11 show that the cannabinoids concentrations determined in this work do not have significant difference from those in CoA. The percentage differences are all below 20%.

when applying the same method on hemp, the method is still robust.

Table 11: Comparison of the cannabinoids concentrations in hemp determined in this work and from CoA

Day 4	Cannabinoids Concentration in Sample (mg/g)								
	CBDA	THCV	CBD	CBG	CBN	Δ9-THC	Δ8-THC	THCA	CBC
UPLC*	63.413	ND	54.502	1.385	0.271	3.081	ND	0.894	3.538
CoA**	67.160	NT	52.300	1.425	0.267	2.987	NT	1.078	3.193
PD***	5.6%	NA	4.2%	2.8%	1.8%	3.2%	NA	17.1%	10.8%

UPLC*: The concentration of each cannabinoid is the average in all hemp samples in day 4.

CoA**: The concentration of each cannabinoid is from the CoA provided by the seller.

PD***: Percentage difference.

Selectivity: Retention Time Study

In general, the retention times of all the nine cannabinoids in this method are stable. Standard deviation of retention time was calculated using all seven calibration standards for each day of validation. The standard deviations of all nine cannabinoids are smaller or equal to 0.02 min (see in Table 12). This demonstrates a good stability of the retention times in this method. The resolution of retention time was calculated using the highest calibration standard (100 ppm) for day 1 to day 3. The resolutions of the peaks are all above 1.3, which demonstrates an acceptable separation.

Table 12: Retention time study for the nine cannabinoids in four different days

	Day 1	Day 2	Day 3	Day 4	AVG	STDEV	Resolution*	Diff. 2 peaks**	2.5% AVG
CBDA	2.16	2.17	2.16	2.17	2.16	0.004			0.05
CBG	2.38	2.39	2.38	2.39	2.38	0.006	3.4	0.22	0.06
CBD	2.51	2.53	2.52	2.53	2.52	0.006	2.0	0.14	0.06
THCV	2.71	2.73	2.72	2.73	2.72	0.006	2.8	0.20	0.07
CBN	3.65	3.67	3.67	3.68	3.67	0.011	11.5	0.94	0.09
Delta9-THC	4.57	4.59	4.59	4.60	4.59	0.015	9.1	0.92	0.11
Delta8-THC	4.72	4.74	4.74	4.76	4.74	0.016	1.3	0.15	0.12
CBC	5.67	5.70	5.70	5.72	5.70	0.020	7.6	0.96	0.14
THCA-A	6.12	6.15	6.14	6.16	6.14	0.015	2.8	0.44	0.15

Resolution*: The resolution of the peak and the previous peak.

Diff. 2 peaks8**: The retention time difference between the peak and the previous peak.

The retention time acceptance window of each cannabinoid was then calculated using the average retention time of the calibration standards in the same run of the samples. The acceptance window is set as the average retention time +/- 2.5% of the average retention time. The retention time acceptance windows on day 1 are given in Table 13 and the windows for the other days can be find in the attached spread sheets.

Table 13: Retention time acceptance window on day 1

	Average	Acceptance Window (Avg ± 2.5%Avg)	Control Limit
CBDA	2.16	2.16 ± 0.05	2.10 - 2.21
CBG	2.38	2.38 ± 0.06	2.32 - 2.43
CBD	2.51	2.51 ± 0.06	2.45 - 2.58
THCV	2.71	2.71 ± 0.07	2.65 - 2.78
CBN	3.65	3.65 ± 0.09	3.56 - 3.74
Δ9-THC	4.57	4.57 ± 0.11	4.45 - 4.68
Δ8-THC	4.72	4.72 ± 0.12	4.60 - 4.84
CBC	5.67	5.67 ± 0.14	5.53 - 5.81
THCA	6.12	6.12 ± 0.15	5.97 - 6.28

Measurement Uncertainty

Measurement Uncertainty for this method is calculate using recovery of the matrix spike samples from 4 different runs (MS3 from day 1, MS from day 2, day 3 and day 4).

Table 14: Cannabinoids Concentration in Matrix Spike samples on 4 different days

Matrix Spike	Cannabinoids Concentration in Matrix Spike (mg/g)								
	CBDA	THCV	CBD	CBG	CBN	Δ9-THC	ΔB-THC	THCA	CBC
Day 1	0.5000	0.4975	0.4909	0.4915	0.4899	0.4879	0.4903	0.4901	0.4849
Day 2	0.5227	0.5103	0.4824	0.4872	0.4854	0.5065	0.5073	0.5023	0.5035
Day 3	0.5073	0.5007	0.4727	0.4741	0.4669	0.4946	0.4956	0.4713	0.4864
Day 4	0.5210	0.5087	0.4810	0.4816	0.4754	0.4979	0.4971	0.4790	0.5049
RSD	2.13%	1.23%	1.55%	1.56%	2.15%	1.56%	1.43%	2.79%	2.17%

Measurement Uncertainty = 2 x RSD at 95% confidence level

Table 15: Measurement Uncertainty for the 9 cannabinoids

	CBDA	THCV	CBD	CBG	CBN	Δ9-THC	Δ8-THC	THCA	CBC
Measurement Uncertainty	4.3%	2.5%	3.1%	3.1%	4.3%	3.1%	2.9%	5.6%	4.3%

Conclusion

This UPLC method is suitable for measuring the nine cannabinoids concentration (CBDA, THCV, CBD, CBG, CBN, Δ9-THC, Δ8-THC, THCA and CBC) in cannabis flower sample.

Reference

1. Guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics, and Veterinary Products, 3rd Edition, US Food & Drug Administration, October 2019

Validation Data Package

All the data, calculation and reports can be found in G:\MCSB\CTLB\Potency\Cannabinoids UPLC Vlidation_Mar2022.