GemmaCert

GemmaCert HPLC Protocol: Analytical Method for Determination of Cannabinoids

ISRAC
Israel Laboratory Accreditation Authority
ISO/IEC 17025

ilac-MRA

1. Equipment, Solvents, Reagents and Devices:

1.1. Solvents, Reagents, Analytical standards and disposable Equipment:

Methanol HPLC grade
Acetonitrile HPLC grade
Formic Acid 99%
Water HPLC grade
Ammonium formate
Sigma (Cerilliant) /Restek analytical standard
50 mL centrifuge tubes
15 mL centrifuge tubes
PTFE Syringe Filter 0.22 μm
0.45 $\mu m\ membrane\ filter$.
Clear and amber glass HPLC vials

1.2. Devices:

Sonicator
Shaker
MERCK 'HITACHI' Lachrome HPLC system (pumps, injector, UV detector)/
MERCK 'HITACHI' Lachrome Elite HPLC system (pumps, autosampler, UV detector)

2. Chromatographic Conditions:

Column: Raptor ARC-18 2.7 µm 150x4.6mm
Guard- Raptor ARC-18 2.7 µm 5x4.6mm
Detector: UV at 228nm
Column temp: $30℃$
Autosampler temp: $10℃$ (N.A for Hitachi)
Flow rate: 1.5 ml/min
Flow mode: Isocratic A: B (25:75)
Injection volume: $5.0\mu L$
Run time: 10.0 min
Mobile phase-Preparation for 1L solution:
Mobile Phase A- 10mM Ammonium Formate and 0.1% Formic Acid Buffer:
Weigh about 0.6 gr of Ammonium Formate, dissolve with 1000ml water and add 1.0 ml of Formic Acid 99%-filter mobile phase A through 0.45 $\mu m\ membrane\ filter$.
Mobile Phase B-0.1% Formic Acid in Acetonitrile:
Add 1.0 ml of Formic Acid 99% into 1000ml of Acetonitrile.
Mobile phases are stable if system suitability can be achieved and not more than 2 weeks at RT.
Diluent: Methanol
Injection wash: Methanol
Pressure: 0-300bar maximum.

Table 2.1:

Time (min)	%A	% B (ACN)	% C (10% ACN in Water)	% D (ACN)
0	25	75	0	0
10	25	75	0	0

3. **Standard preparation:**

Stock sol 1:

Accurately transfer 50μL of each standard (conc.: 1 mg/mL =1000ppm) into the same HPLC vial add 100µL diluent, Mix with vortex. This is stock solution 1 (conc. 100ppm). Dilute next standards with $V_{Stock\ solution}$ and $V_{diluent}$ according to following table, after each dilution mix vial with vortex:

Table 4.1:

Concentration [ppm]	$V_{Stock\ solution\ (100ppm)}$	$V_{diluent}$	Total volume:
100ppm stock sol 1			500µL
50ppm	$100\mu L$	$100\mu L$	200µL
25ppm	$50\mu L$	$150\mu L$	200µL
10ppm-stock solution no.2	$150\mu L$	$600 + 750\mu L$	1500µL
Use stock solution no.2 for next dilutions:			
5ppm	$100\mu L$	$100\mu L$	200µL
2.5ppm	$50\mu L$	$150\mu L$	200µL
1ppm	$50\mu L$	$450\mu L$	500µL

*Any other combination of dilutions may be used if final conc. Is maintained.

4. **System suitability:**

a. New 7[1] points calibration curve must be injected as system suitability before any new project unless QC solution (10 ppm) is injected as system suitability and Total %RSD when taking into consideration last QC solution from previous calibration curve and all following QC solution injections during last run is under 2.5% for all the 8 cannabinoids[2].

1- One point from the calibration curve can be removed if necessary (excluding 100 ppm) to achieve linearity.

2- System suitability must be checked prior to samples injection, when %RSD is not met, a new calibration curve must be injected.

b. Calibration's curve 10ppm solution will be injected in triplicate to demonstrate the system's reproducibility and stability. %RSD for all 8 cannabinoids must be NMT 2.5%.

c. Retention time and order of elution of main peaks is according to next table:

Table 4.1:

No.	Cannabinoids Name	Approx. Retention time (min)
1	CBDA	**2.7**
2	CBGA	**2.9**
3	CBG	**3.0**
4	CBD	**3.1**
5	THCV	**3.4**
6	CBN	**4.6**
7	Δ^{9}-THC	**5.8**
8	THCA	**7.6**

d. Tailing for all the peaks should be NMT 2.0 and NLT 0.8. Tailing may be examined for 10ppm and higher standard solutions.

e. $R^2 for\ all\ cannabinoids\ \ must\ \ be\ NLT\ 0.999$

5. Sample preparation:

5.1. Extraction: Grind the flower using suitable grinder with at least three rotations and transfer about 0.2 gr of grinded flower into a 50-mL centrifuge tube (For big flowers weigh in duplicates or triplicates). Dissolve the sample by Accurately adding 10.0 mL of diluent into the centrifuge tube. Sonicate for 15 min in iced water and then shake for another 15 min. Filter about 1.5mL of the sample solution into an amber HPLC vial through 0.22 PTFE membrane filter. This is stock sample solution. 0.02 gr/ml

5.2. dilutions:

5.2.1. Dilution factor 100: transfer 100 μL of stock sample solution and 9.9 mL of diluent into a 15-mL centrifuge tube, mix with vortex and pipet a portion of the solution into a HPLC vial. 0.0002 gr/ml

5.2.2. Dilution factor 8: transfer into a HPLC Vial 130µL of stock sample solution with 910µL diluent and mix with vortex. 0.0025 gr/ml

5.3. Oil Extraction:

For full oil extraction process follow OIL00001 work instructions.
For analyses of oil extract, extract as described in section 6.1 then, dilute the extracts by factor 100 (as in section 6.2.1)-this is intermediate solution. Further dilute the samples by factor 5:
Accurately transfer 200 µL of the intermediate solution + 800 µL MeOH to an HPLC vial, Mix. 0.00004 gr/ml

6. Sample for LOD test preparation:

6.1. For each cannabis species prepare sample for LOD in triplicate. Weigh an empty weighing vessel, zero the balance and weigh about 2.0 gr of grind cannabis collected from each species flowers. Place the weighed vessels with samples, inside an oven heated to 70°C for 72 hours. (According to LOD00001 procedure)

6.2. Weigh the weighing vessels with dried sample and calculate %LOD:

$$\%LOD = \frac{W1 - W2}{Sample\ weight} * 100\%$$

$W1 - Vessel\ weight\ before\ drying$
$W2 - Vessel\ weight\ after\ drying$

6.3. %LOD can be also tested by using sartorius Moisture analyzer according to SOP00004 and LOD00001 procedures.

7. HPLC PROCEDURE:

7.1. Inject diluent at least twice to identify system peaks and any interferes with the method peaks of interest (appendix 1).
7.2. Inject calibration curve or QC solution and check system suitability according to section 5 (appendix 2).
7.3. Inject samples, if both concentrations are injected together the lower concentration samples should be injected first.
7.4. After every 20 samples inject diluent and then QC solution, Inject QC solution in the end of the sequence as well. % RSD for all QC injection must be NMT 2.5%[1,2].
7.5. In the end of work use a proper program for washing the column to manufacture storage instructions.

1- If % RSD was Higher than 2.5%, wash column with 100% ACN (1.5 ml/min). Then set again to A: B (25:75) mobile phase. Wash autosampler and inject MeOH in triplicate and one QC (10ppm solution). Check %RSD of this injection and 3 replicates from calibration curve. If %RSD is less than 2.5% work is valid or can be continued in case of injecting part 2.

2- Removing one QC solution- if one of the QC solutions injected during the sequence (**excluding the one injected in the end)** is clearly exceptional (by peak area) than all the others, which demonstrate a clear trendline, this solution may be removed for %RSD calculations. This may occur due to pressure drop or some failure in injection process.

8. Integration:

8.1. Ignore any peak which is not injected in the calibration curve.
8.2. Integration may be inhibited before elution of CBDA and after the elution of THCA.
8.3. Minimum area should be no bigger than the area of the smallest peak in 1.0ppm standard solution injection.

9. Calculations and reports:

10.1 Calculate sample concentration (ng/µL) by validated calculating program or by HPLC software.

Calculate %W according to following equation:

$$\%W = \frac{\frac{Amount[ng]}{inj\ vol[\mu L]} \cdot Extr.vol[ml] * 10^3[ml \rightarrow \mu L] \cdot DF \cdot 100\%}{M * 10^9[gr \rightarrow ng] \cdot Purity}$$

$Extr.vol - extraction\ vol\ [ml]$

$DF - dilution\ factor$

$M - dried\ sample\ weight\ [gr]\ M = Sample\ weight - (\frac{Sample\ weight \cdot \%LOD}{100})$

$Purity$–standard potency/Assay

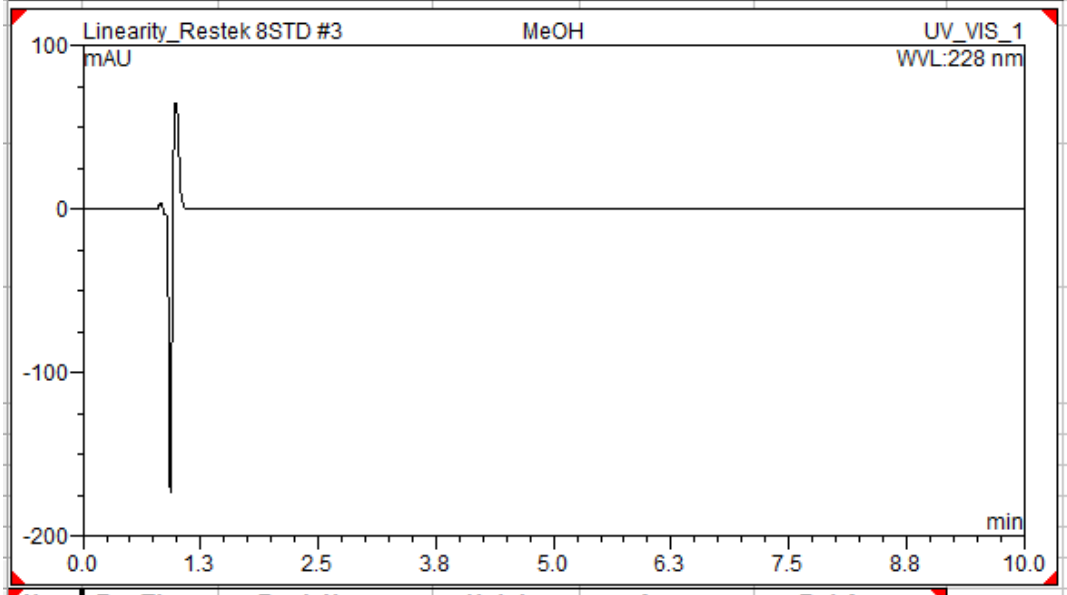
10.2. Report all relevant data to system suitability, chromatograms, sample concentration and %W for all samples.

Appendix 1:

Blank Chromatogram:

3 MeOH

Sample Name:	**MeOH**	*Injection Volume:*	**5.0**
Vial Number:	**BA1**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**228**
Control Program:	**Restek ARC-18 20_06_19**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Restek ARC-18_8STD**	*Dilution Factor:*	**1.0000**
Recording Time:	**23/6/2019 11:09**	*Sample Weight:*	**1.0000**
Run Time (min):	**10.01**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %
n.a.	n.a.	CBDA	n.a.	n.a.	n.a.
n.a.	n.a.	CBGA	n.a.	n.a.	n.a.
n.a.	n.a.	CBG	n.a.	n.a.	n.a.
n.a.	n.a.	CBD	n.a.	n.a.	n.a.
n.a.	n.a.	THCV	n.a.	n.a.	n.a.
n.a.	n.a.	CBN	n.a.	n.a.	n.a.
n.a.	n.a.	THC-9	n.a.	n.a.	n.a.
n.a.	n.a.	THCA	n.a.	n.a.	n.a.

No.	Ret.Time min	Area uAU*Sec	K'	Resolution(EP)	Asymmetry(EP)
n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

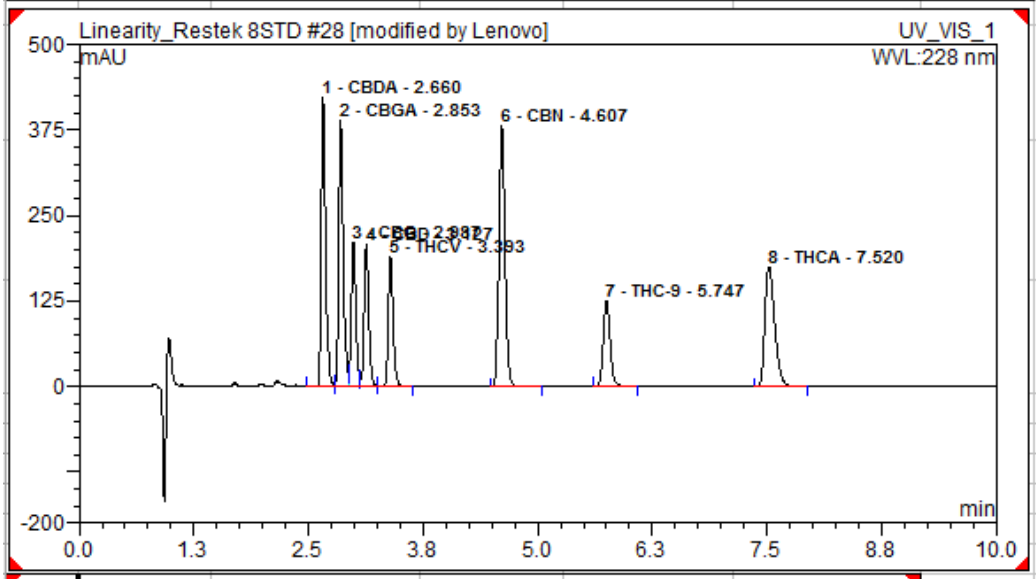
Appendix 2:

100ppm Chromatogram:

-for CBG Asymmetry see validation report -manual calculation.

28 10STD 100ppm-3

Sample Name:	10STD 100ppm-3	*Injection Volume:*	5.0
Vial Number:	RC7	*Channel:*	UV_VIS_1
Sample Type:	unknown	*Wavelength:*	228
Control Program:	Restek ARC-18 20_06_19	*Bandwidth:*	n.a.
Quantif. Method:	Restek ARC-18_8STD	*Dilution Factor:*	1.0000
Recording Time:	23/6/2019 15:32	*Sample Weight:*	1.0000
Run Time (min):	10.01	*Sample Amount:*	1.0000



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %
1	2.66	CBDA	422.653	23.507	16.22
2	2.85	CBGA	388.841	22.394	15.45
3	2.99	CBG	209.719	12.567	8.67
4	3.13	CBD	208.342	12.609	8.70
5	3.39	THCV	190.517	11.882	8.20
6	4.61	CBN	382.275	29.226	20.17
7	5.75	THC-9	123.810	11.297	7.80
8	7.52	THCA	173.945	21.420	14.78

No.	Ret.Time min	Area uAU*Sec	K'	Resolution(EP)	Asymmetry(EP)
1	2.66	1410401.336	2.06	2.19	1.34
2	2.85	1343662.618	2.28	1.46	1.34
3	2.99	754042.016	2.43	1.51	n.a.
4	3.13	756540.058	2.59	2.82	1.18
5	3.39	712901.962	2.90	11.33	1.28
6	4.61	1753574.540	4.30	8.79	1.20
7	5.75	677810.276	5.61	10.65	1.21
8	7.52	1285193.844	7.64	n.a.	1.44

Change control table:

Edition	Details of change	Effective date
1.0->2.0	Section 2, line 12 addition of 'ml' units	03.06.20
2.0 => 3.0	Section 1.2, changed Dionex to Lachrome Elite HPLC	19.10.20

Document approval table:

Position	Name	Signature
Lab Manager	Olga Pendo	
CEO	Guy Setton	