

Analytical Monograph Cannabis Flos (flowers / granulated)

OMC / Farmalyse BV Version 7.1 / November 28, 2014

Release Testing of Cannabis Flos

Introduction

This monograph describes the release testing of Cannabis Flos (flowers / granulated), specifically the varieties: Bedrocan, Bedrobinol, Bediol, Bedica, Bedrolite and Bedropuur. The test parameters are:

1. Appearance
2. Foreign Material
3. Fineness
4. Identification A: Microscopic Properties
5. Identification B: TLC
6. Microbiological Contamination
7. Aflatoxins
8. Pesticides
9. Heavy Metals
10. Loss On Drying
11. Assay and Related Substances

Sample Preparation

The ungrinded sample will be used for test numbers 1, 2, 3, 6 and 7 (except if the sample material is already in granulated form). The remaining sample will be grinded using a simple kitchen blender until the material is about 5 mm in diameter, homogenised and subsequently used for the remaining tests. Please note that the LOD test has to be carried out on the same day as the samples for the test Assay and Related Substances are prepared.

List of Abbreviations

Δ 9-THC:	Δ 9-Tetrahydrocannabinol
Δ 8-THC:	Δ 8-Tetrahydrocannabinol
THCA:	Tetrahydrocannabinol Acid
CBD:	Cannabidiol
CBDA:	Cannabidiol Acid
CBG:	Cannabigerol
CBN:	Cannabinol
cm:	centimeter
EP:	European Pharmacopoeia
ICP-OES:	Inductively Coupled Plasma – Optical Emission Spectroscopy
LLOQ:	Lower Limit Of Quantitation
LOD:	Loss On Drying
mg:	milligram
min.:	minute(s)
mL:	milliliter
mm:	millimeter
μ L:	microliter
NLT:	Not Less Than
NMT:	Not More Than
PTFE:	Poly Tetra Fluoro Ethylene
RF:	Response Factor
rpm:	rotations per minute
RRT:	Relative Retention Time
RSD:	Relative Standard Deviation
S/N:	Signal-to-Noise ratio
SST:	System Suitability Test
TAMC:	Total Aerobic Microbial Count
TYMC:	Total Yeast and Molds Count
TBA:	2-t-Butyl-Anthraquinone
TLC:	Thin Layer Chromatography
v/v:	volume / volume



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1. Appearance

Bedrocan, Bedrobinol,
Bedropuur:

Brown green clustered flowers of 1.5 to 3 cm length with a characteristic smell.

Bediol, Bedica and
Bedrolite:

Brown green granulate of the flowers (about 5 mm) having a characteristic smell.

2. Foreign Material

Bedrocan, Bedrobinol,
Bedropuur:

The sample material is free from stalks, insects and other vermin.

Bediol, Bedica and
Bedrolite:

The sample material is free from insects and other vermin.

3. Fineness

Bedrocan, Bedrobinol,
Bedropuur:

Macroscopic inspection of the ungrinded material does not show leaves shooting out more than 20% of the length of the flowers. Moreover, the stalks are cut away directly under the bottom flowers of the inflorescence.

Bediol, Bedica and
Bedrolite:

Stalks are not longer than 2.0 cm and only 20% of the stalks is between 1.5 and 2.0 cm.

4. Identification A: Microscopic Properties

4.1 Reagents

Chloral hydrate solution: A solution of 80 grams Chloral hydrate in 20 mL Demi water.

4.2 Execution

Prepare a Chloral hydrate preparation by adding a few drops of the Chloral hydrate solution to a spatula tip of plant material and shortly cooking it on a little flame. Under the microscope gland hairs are mainly observed.

5. Identification B: TLC

5.1 Reagents

Petroleum ether (40-60 fraction)

Diethyl ether

Fast Blue B Salt (stabilised with Zinc chloride); CAS number: 14263-94-6

Ethanol (absolute)

Methanol

Bedrocan reference extract

Bedrobinol reference extract

Bediol reference extract

Bedica reference extract

Bedrolite reference extract

Bedropuur reference extract



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5.2 Execution

TLC-plate: Silicagel F₂₅₄ (Merck art. nr. 5715)
Eluent: Petroleum ether : Diethyl ether = 40 : 10 (v/v). Saturate the TLC chamber.
Travel distance of solvent front: 10 cm.
Spray agent: A solution of 1 gram Fast Blue B Salt in a mixture of 50% Ethanol in Demi water.

System Suitability

Use the Bediol reference extract for the SST. The red coloured spot (THCA) and the orange coloured spot (CBDA) must be well separated.

Analysis

Sample solution A:

Use Sample solution 1A from the test "Assay and Related Substances" (see Section 11).

Standard solution A:

Use the reference extract that matches the variety to be analysed in the test "Assay and Related Substances" (see Section 11).

Apply 10 µL of the sample solution and standard solution to the TLC plaat in a round spot of max. 5 mm width. Do not apply at once, but dry in between. Place the plate in the TLC chamber containing eluent and let the solvent front develop about 10 cm. Dry the plate in the air, apply spraying agent and if necessary warm the plate with a laboratory heater to enhance the visibility of the spots. Observe the plate in normal light.

Assessment of the plate

Bedrocan, Bedrobinol, Bedica and Bedropuur:

The main spot in Sample solution A is coloured red (THCA) and has an RF value and intensity equal to those of the corresponding reference extract. The chromatogram of Sample solution A may show a red-violet zone corresponding to Δ^9 -THC.

Bedrolite:

The main spot in Sample solution A is coloured orange (CBDA) and has an RF value and intensity equal to those of the corresponding reference extract.

Bediol:

In addition to the spots mentioned before, an orange coloured spot is observed corresponding with the spot from the reference extract of Bediol (CBDA).

Location of the spots:

Top of the plate	
Orange zone (CBD) Red-violet zone (Δ^9 -THC)	
Red zone (THCA) Orange zone (CBDA)	Red zone (THCA) Orange zone (CBDA)
Standard solutions	Sample solution A

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6. Microbiological Contamination

These tests are executed on 10 grams ungrinded material. The material that has not undergone germ reducing treatment is tested for:

- TAMC
- TYMC
- Total combined count (TAMC + TYMC)
- Enterobacteriaceae and gram negative bacteria
- *Pseudomonas aeruginosa*
- *Staphylococcus aureus*

7. Aflatoxins

This test is executed on 5.0 grams ungrinded material. The analysis is according to the current EP monograph "Determination of aflatoxins B₁, B₂, G₁ and G₂ in herbal drugs (2.8.18)". The specification is NMT 4 µg per kg.

8. Pesticides

Pesticides are tested according to EP monograph 2.8.13.

It should be noted that not all EP Pesticides are being analysed with the GC-MS method. For that reason the following text is added to the CoA: "The following components are not being analysed with the current method: Bromide inorganic, Dithiocarbamates, Fenchlorophos, Methacriphos, S-421".

9. Heavy metals

This test is executed on 5 grams grinded material using different atomic absorption and emission techniques.

Lead:	max. 20.0 ppm (ICP-OES)
Cadmium:	max. 0.5 ppm (ICP-OES)
Mercury:	max. 0.5 ppm (Combustion Atomic Absorption)
Arsenic:	Indicative (ICP-OES)
Nickel:	Indicative (ICP-OES)
Zinc:	Indicative (ICP-OES)

10. Loss on drying

This test is executed on 0.500 gram of the grinded sample material using EP monograph 2.3.32 method C. It is heated during 24 hours at 40°C above Phosphorous Pentoxide under vacuum. The specification is: NMT 10.0%.

11. Assay and Related Substances

11.1 Reagents

Ethanol (absolute)
Acetonitrile
Formic Acid
Milli-Q Water

11.2 Equipment

UPLC-column: Waters Aquity C18, 1.7 µm, 2.1 x 150 mm
Column temperature: 30 °C
Tray temperature: 8 °C

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Mobile phase:	0.1 % Formic Acid in Acetonitrile (A) 0.1 % Formic Acid in Milli-Q Water (B)
Solvent / Needle wash:	A : B = 70 : 30 (v/v)
Centrifuge:	Eppendorf, Model 5810 (or equivalent)
Gradient:	t (min.) A (%) B (%)
	0 70 30
	6.00 70 30
	10.50 100 0
	10.70 100 0
	11.00 70 30
	12.50 70 30
Flow:	0.4 mL / min.
Injection volume:	10 µL
Detection (UV):	228 nm
Run time:	12.5 min.

11.3 Execution

Sample Solutions (fresh material)

Weigh 1000 mg of the grinded sample material in a "Falcon tube" and shake (ca. 300 rpm) during 15 minutes with 40 mL Ethanol and centrifuge (3000 rpm). Transfer the clear upper layer into a 100 mL volumetric flask. Repeat this step two times with 25 mL Ethanol and make up the solution to the mark with Ethanol. Filter the Sample solution over a 0.45 µm PTFE filter. Pipet 1.0 mL of the filtrate in a volumetric flask of 10 mL and make up to volume with Solvent (*Sample Solutions 1A M1 and 1A M2*). Dilute Sample Solution 1A a factor 10 with Solvent (*Sample Solutions 1B M1 and 1B M2*).

Standard Solutions

Weigh accurately about 50 mg TBA standard in a volumetric flask of 100 mL, add 25 mL Solvent and dissolve. Make up to volume with Solvent. Dilute 1.0 mL to 50.0 mL with Solvent (S1+S2; 0.01 mg / mL).

LLOQ Solution

Dilute the Standard Solution TBA with a concentration of 0.01 mg / mL to a concentration of 0.002 mg / mL by pipetting 5.0 mL of the Standard Solution TBA in a volumetric flask of 25.0 mL and make up to volume with Solvent.

Resolution Solution

Make a mixture containing Δ9-THC and Δ8-THC, wherein the ratio of Δ9-THC / Δ8-THC should be 0.04 mg / mL and 0.0025 mg / mL respectively. This solution can be prepared for instance by the following method. Dilute 200 µL Δ9-THC Stock Standard Solution (*i.e.* 2 mg / mL Δ9-THC in Ethanol) and 1000 µL Δ8-THC Stock Standard Solution (*i.e.* 0.025 mg / mL Δ8-THC in Ethanol) in a volumetric flask of 10 mL and make up to volume with Solvent.

Reference Extract Solution

It should be noted that each variety has its own reference extract.
Prepare a dilution (factor 5) from the reference extract by pipetting 50 µL of the reference extract (corresponding to the sample) in a vial with insert and dilute with 200 µL Solvent.

Injection Sequence

Prepare the following injection sequence:

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Injection Nr	Vial content	Purpose
1	Blanc Solution	SST
2	Blanc Solution	SST
3	LLOQ Solution	SST
4	Resolution Solution	SST (Resolution)
5	Standard Solution S1 – Injection 1	SST (RSD)
6	Standard Solution S1 – Injection 2	SST (RSD)
7	Standard Solution S1 – Injection 3	SST (RSD)
8	Standard Solution S1 – Injection 4	SST (RSD)
9	Standard Solution S1 – Injection 5	SST (RSD)
10	Standard Solution S1 – Injection 6	SST (RSD)
11	Standard Solution S2 – Injection 1	SST (Recovery)
12	Standard Solution S2 – Injection 2	SST (Recovery)
13	Reference Extract Solution	Check Sample against Reference
14	Sample Solution 1A M1	Assay & Related Substances
15	Sample Solution 1A M1	Assay & Related Substances
16	Sample Solution 1A M1	Assay & Related Substances
17	Sample Solution 1A M1	Assay & Related Substances
18	Standard Solution S1 – Injection 1	Check for drift of Std during analysis

11.4 System Suitability Test

- The RSD of 6 replicate injections of Standard Solution S1 (peak area TBA) is $\leq 2.0\%$;
- Standard Solution S2 has a Recovery of 98.0% to 102.0% when calculated against Standard Solution S1;
- The Resolution between the peaks of $\Delta 9$ -THC en $\Delta 8$ -THC in de Resolution Solution should be ≥ 1.2 ;
- The TBA peak in the LLOQ Solution has a S/N value of ≥ 10 ;
- The chromatographic profile of Sample Solution 1A must resemble that of the corresponding Reference Extract.

11.5 Calculation Assay THCA (and CBDA in Bediol and Bedrolite)

Sample solutions 1B M1 and 1B M2 are used to calculate the total content of THCA on dried sample material according to the formula displayed below. See Table 1 for the corresponding Response Factors. When testing the varieties Bediol and Bedrolite, also the total content of CBDA has to be calculated.

$$\text{THCA (\%)} = 100 \times \frac{A_m \times V_m \times W_s \times V_f}{R_f \times A_s \times V_s \times W_m} \times \frac{100}{(100 - LOD)}$$

$$\text{THC total equivalents (\%)} = \% \text{THCA} \times 0.877 + \% \text{THC}$$

In which:

- A_{m1B} : Area of Sample (for Assay)
 A_{m1A} : Area of Sample (for Related Substances)
 V_m : Volume of Sample (mL)

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Ws:	Weight of TBA reference standard (mg)
RF:	Response Factor against TBA (see Table 1)
As:	Area of TBA reference standard
Vs:	Volume of TBA reference standard (mL)
Wm:	Weight of Sample (mg)
Vf:	Dilution factor (10 or 100)
LOD:	Loss On Drying (see Section 10)
0.877:	Factor for the conversion of the Molecular Weights

11.6 Calculation of Related Substances

Calculate the Related Substances (total equivalents) from Sample Solution 1A M1 and 1A M2 on dried basis using the example calculation shown above. See Table 1 for the corresponding Response Factors.

Concentrations less than 0.05% shall be reported as NMT 0.05%.

Table 1: RRTs and RFs for UPLC.

Component	RRT	RF
CBDA	0.78	1.91
CBD	0.92	1.00
TBA	1.00	1.00
CBN	1.43	2.30
THC	1.62	0.91
CBNA	1.67	1.50
THCA	1.82	1.66

12. Literature

1. Monograph "BMC/Farmalyse ter vrijgiftecontrole van Cannabis Flos (Hennepbloemen); variëteiten Bedrocan, Bedrobinol en Bediol; versie 7.0." (in Dutch).
2. EP 01/2012:1433 Herbal drugs; for the tests LOD and Heavy Metals.
3. "Validatie gehaltebepaling m.b.v. UPLC uitgevoerd door Farmalyse" (rapport UPLC-0512-1); in Dutch.
4. "Pesticiden: Validatie uitgevoerd door TNO Voeding met als referentie ASC-2004/0941rev1-mol."; in Dutch.
5. Validation of the analytical method for the determination of the Cannabis Flos variety Bedica and the determination of the response factors of the cannabinoids Δ^9 -THC, CBD, CBN, THCA, CBDA and CBNA. VWS 2012-013 *version 2*. April 18, 2013.
6. Determination of the Cannabis Flos varieties Bedrocan and Bedica on the Thermo (U)HPLC at Farmalyse B.V. VWS, September 2013.

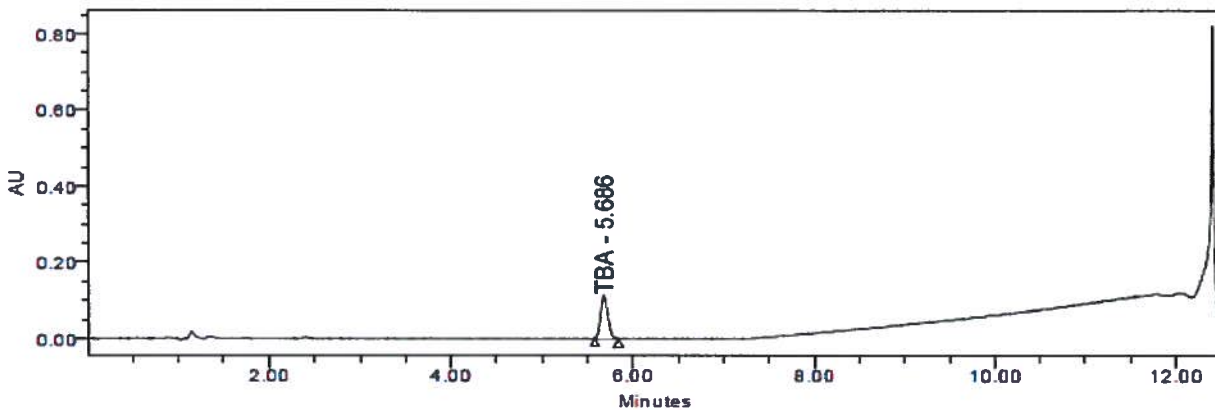
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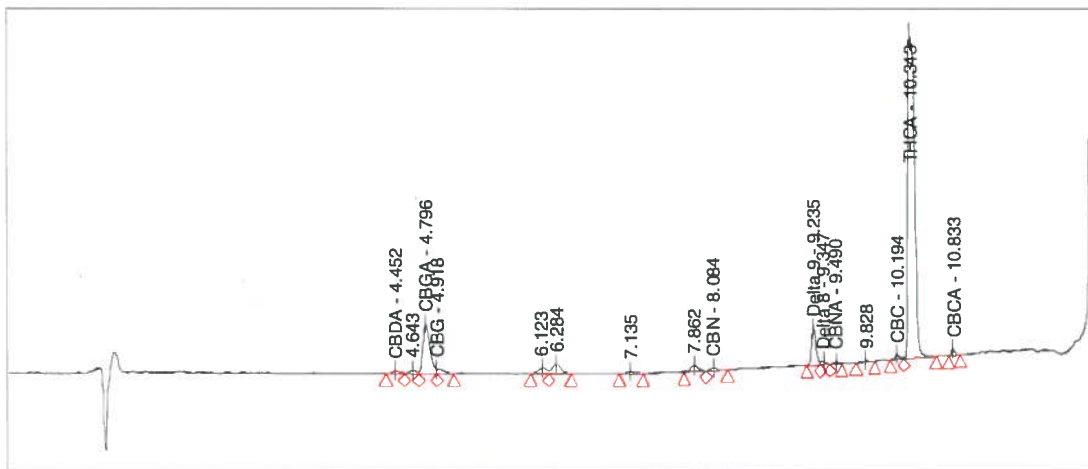
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Annex

This annex contains various example chromatograms.



Chromatogram of Standard Solution

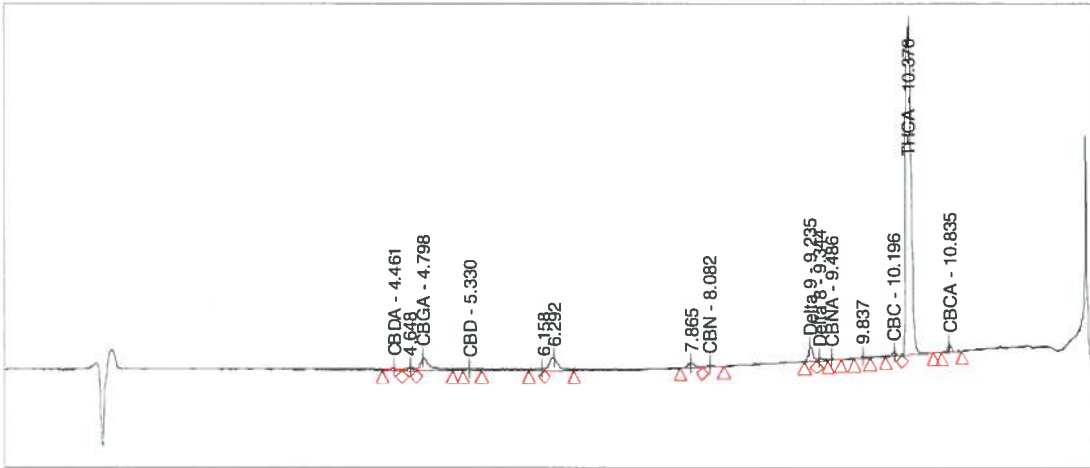


Chromatogram of Reference Extract Bedrocan

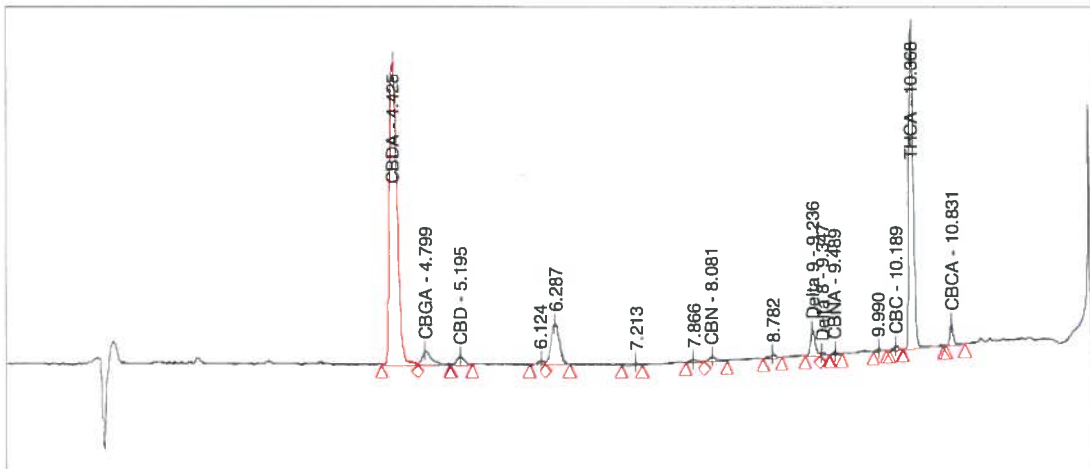
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Chromatogram of Reference Extract Bedrobinol



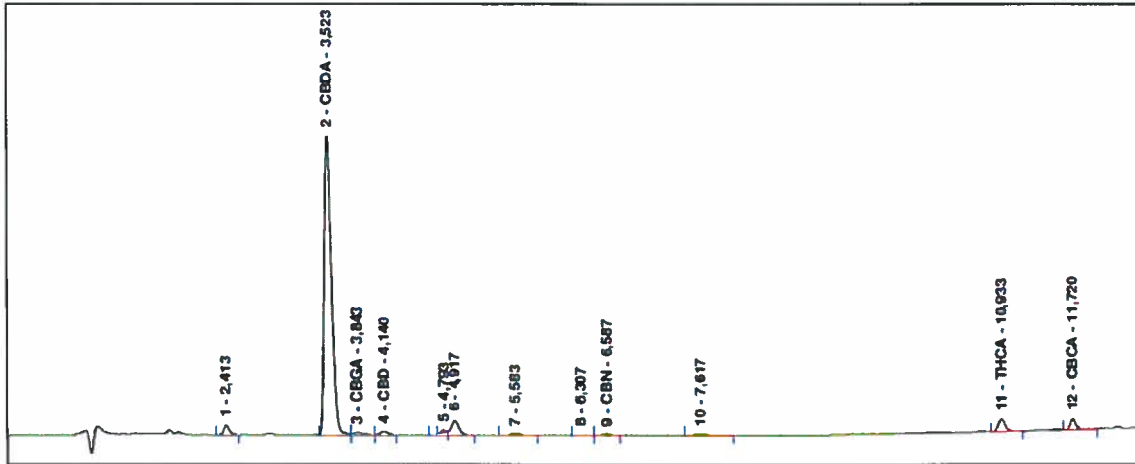
Chromatogram of Reference Extract Bediol



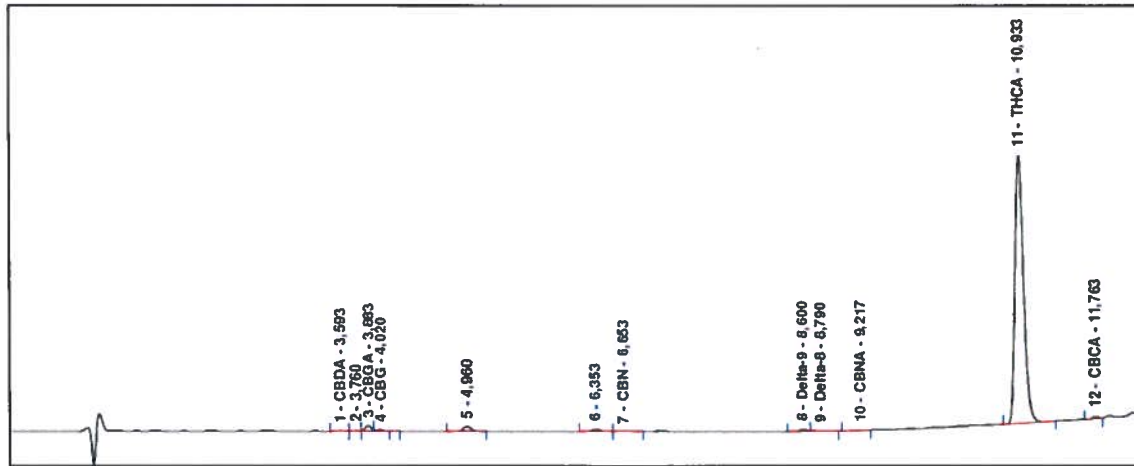
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Chromatogram of Reference Extract Bedrolite



Chromatogram of Reference Extract Bedropuur