

PharmaHemp d.o.o., Koprška ulica 106C, SI-1000 Ljubljana, Slovenia		
QUANTITATIVE DETERMINATION OF CANNABINOIDS BY REVERSE PHASE CHROMATOGRAPHY		
Issue date:	Expiration date:	Code: MET-LAB-001
		Revision No.: 09
		Appendixes: 1
Author: Janja Ahej	Reviewer: Dr. Boštjan Jančar	Approver: Tine Lavrič

TRAINING/FAMILIARITY

Scope of training	
Scope of familiarity	LAB

DEFINITIONS AND ABBREVIATIONS

ACN	Acetonitrile
CRM	Certified reference material
GC	Gas chromatography
HPLC	High Performance Liquid Chromatography
LAB	Analytical Laboratories
LC	Liquid chromatography
MPA	Mobile phase A
MPB	Mobile phase B
RPC	Reverse Phase Chromatography
SOP	Standard operative procedure
SST	System suitability tests
QA	Quality assurance
QCS	Quality control sample

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QUANTITATIVE DETERMINATION OF CANNABINOIDS BY REVERSE PHASE CHROMATOGRAPHY

1. PURPOSE

This laboratory procedure describes the quantitative determination of cannabinoids using reverse phase chromatography on HPLC systems. The description includes sample preparation and handling as well as detailed description of experimental and data processing parameters / settings. The procedure defines 3 previously independent analytical methods which are described with separate instructions/procedures. When necessary, the conformation of Δ -9-THC and Δ -9-THCA reporting should be checked following the specific procedure instructions (see Chapter 9 for more information).

The method enables the determination of 16 cannabinoids (see Table 4) in a wide variety of samples. The given results of the concentration for each of the cannabinoid are calculated using calibration curve from the known concentrations of cannabinoids in certified reference standard materials (CRMs). Quality samples control samples are used for the evaluation of validity of each analysis, in accordance to the procedure SOP-LAB-001 - Zagotavljanje veljavnosti rezultatov.

In addition, with the method MET-LAB-001 the Total THC can be calculated following the following equation:

$$\text{Total THC}^* = \Delta\text{-9-THC} + 0.877 * \Delta\text{-9-THCA (in \% w/w)}$$

* Assuming complete decarboxylation of Δ -9-THCA, total THC denotes the sum of % w/w Δ -9-THCA multiplied by 0.877 and % w/w Δ -9-THC. The equation for Total THC was determined based on commission regulation (EU) 2022/1393 from 11th of August 2022; amending Regulation (EC) No 1881/2006.

All the procedures described in this document are validated, following the validation protocols:

- VP-01 - Analytical method validation protocol for quantitative determination of cannabinoids by reverse phase chromatography
- VP-005 - Analytical method validation protocol for method MET-002 -01
- VP-011 - Analytical method validation protocol for method MET-003 -01
- VP-013 - Analytical method validation protocol for conformation of Δ -9-THC and Δ -9-THCA

The validation results are presented in the validation reports:

- RP-VP-004 - Analytical method validation report for method MET-001
- RP-VP-007 - Analytical method validation report for method MET-002-01
- RP-VP-011 - Analytical method validation report for method MET-003-01
- RP-VP-LAB-22-003 - Analytical method validation report for conformation of Δ -9-THC and Δ -9-THCA

The methods were initially validated for 10 cannabinoids (CBDV, CBD, CBG, THCV, CBN, Δ -9-THC, CBC, CBDA, CBGA, Δ -9-THCA) and re-validation was performed – first for 2 (Δ -8-THC and CBL) and then for 3 additional cannabinoids (CBV, CBCA and CBT); see validation reports:

- RP-VP-011 - Analytical method validation report for method MET-003-01
- RP-VP-LAB-22-002 - Analytical method validation report for quantitative determination of CBV, CBCA and CBT

Since 2022, the laboratory reports cannabinoid CBE as a non-accredited activity. In 2024 re-validation with CBE CRM was performed for the purpose of accredited reporting of additional cannabinoid CBE; see validation report:

- RP-VP-LAB-24-001 - Analytical method validation report for quantitative determination of CBE-01

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2. RESPONSIBILITIES

All the employees within analytical laboratory are obligated to use this laboratory procedure in accordance with the instruction given within for all the samples received.

Table 1: List of responsibilities

Code	Role	Responsible for
ALT	Analytical Laboratory Technician	Using this laboratory procedure.
JLM	Junior Laboratory Manager	Using this laboratory procedure.
LAB/MAN	Analytical Laboratory Manager	The preparation, evaluation and maintenance of this laboratory procedure.
CDO/LAB	Chief Laboratory Officer	Reviewer of this laboratory procedure.
QA	Quality assurance	Approval of this laboratory procedure.

3. THEORETICAL PRINCIPLE

High performance liquid chromatography (HPLC) has recently become the method of choice for the quantitative determination of cannabinoids in plant materials and cannabis products. In contrast to GC, LC based techniques do not encounter decomposition of the sample as they work at room temperature allowing the direct analysis of cannabinoid acids groups in the extracted samples. The most common used columns are based on reverse phase (RP) C₁₈ stationary phases - thus the name RPC is used. The great advantage in the use of such columns lies in fast analyses and high separation efficiency.

4. HANDLING WITH CHEMICALS AND REAGENTS, SAFETY INSTRUCTIONS

When handling with chemicals and reagents safety precautions on safety data sheets of materials used must be followed. The analyst must use the protective equipment described in the table below.

Table 2: Safety precautions when handling with chemicals and reagents

Substance	Danger	Precautions
Acetone	Highly flammable liquid and vapor. Causes severe eye irritation. May cause drowsiness and dizziness.	Wear protective gloves/protective clothing/eye protection. When aerosol formation or mist occurs wear suitable protective mask. In case of contact with eyes, rinse thoroughly with clean running water for at least 10 minutes.
Acetonitrile	Highly flammable liquid and vapor. Harmful if swallowed, in contact with skin or if inhaled. Causes severe eye irritation.	Wear protective gloves/protective clothing/eye protection. When aerosol formation or mist occurs wear suitable protective mask. In case of contact with eyes, rinse thoroughly with clean running water for at least 10 minutes. If exposed or concerned: Get medical advice/attention.
Ethanol	Highly flammable liquid and vapor. Causes severe eye irritation.	Wear protective gloves/protective clothing/eye protection. In case of contact with eyes, rinse thoroughly with clean running water for at least 10 minutes.
Formic acid	Highly flammable liquid and vapor. Corrosive to metal. Harmful and toxic if swallowed and may cause respiratory irritation. Causes severe skin burns and eye damage.	Wear protective gloves/protective clothing/eye protection/face protection. When aerosol formation or mist occurs wear suitable protective mask. In case of contact with eyes or skin, rinse thoroughly with clean running water for at least 10 minutes. If exposed or concerned: Get medical advice/attention.
Methanol	Highly flammable liquid and vapor. Harmful if swallowed, in	Wear protective gloves/protective clothing/eye protection. When aerosol formation or mist occurs

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Substance	Danger	Precautions
	contact with skin or if inhaled. Causes severe eye irritation.	wear suitable protective mask. In case of contact with eyes, rinse thoroughly with clean running water for at least 10 minutes. If exposed or concerned: Get medical advice/attention.
Isopropanol	Highly flammable liquid and vapor. Causes severe eye irritation. May cause drowsiness and dizziness.	Wear protective gloves/protective clothing/eye protection. When aerosol formation or mist occurs wear suitable protective mask. In case of contact with eyes, rinse thoroughly with clean running water for at least 10 minutes.

5. CHEMICALS AND REAGENTS, CRMs, CONSUMABLES AND EQUIPMENT/SOFTWARE

The stated chemicals and reagents, certified reference materials (CRMs) and consumables were tested and proven to be suitable for the current procedure. All the materials are listed in SPC-LAB-001 - Analitski laboratoriji. Other suitable chemicals, reagents and CRMs of the same quality and performance can be used but the validation study needs to be performed to confirm the suitability of new materials. The new materials can be used only after the suitability is confirmed in validation study. Also, other suitable consumables of the same quality and performance can be used. If different consumables are used their suitability can be confirmed through the validity of each analysis using quality control samples.

Table 3: List of chemicals

Chemicals / reagents	Producer and order number
Acetonitrile	e.g.: Fisher Scientific, 10660131; LabExpert; KE00.0174.2500
Methanol	e.g.: J.T. Baker, 8402; Carlo Erba Reagents, 525102
Isopropanol	e.g.: J.T. Baker, 9095
Formic acid	e.g.: Carlo Erba Reagents, 405824
MQ-Water	e.g.: Adrona, B30 HPLC
MCT oil	e.g.: KEFO, 580731

Table 4: List of certified reference standard materials (CRMs)

CRM	Producer and order number
Cannabidiol (CBD)	e.g.: Sigma-Aldrich; C-045-1ML
Cannabidivarin (CBDV)	e.g.: Sigma-Aldrich; C-140-1ML
Cannabigerol (CBG)	e.g.: Sigma-Aldrich; C-141-1ML
Cannabinol (CBN)	e.g.: Sigma-Aldrich; C-046-1ML
Cannabichromene (CBC)	e.g.: Sigma-Aldrich; C-143-1ML
Cannabichromene (CBL)	e.g.: Sigma-Aldrich; T-005-1ML
Delta-9-tetrahydrocannabinol (Δ -9-THC)	e.g.: Sigma-Aldrich; T-094-1ML
Delta-8-tetrahydrocannabinol (Δ -8-THC)	e.g.: Sigma-Aldrich; C-144-1ML
Tetrahydrocannabivarin (THCV)	e.g.: Sigma-Aldrich; C-142-1ML
Cannabidiolic acid (CBDA)	e.g.: Sigma-Aldrich; T-093-1ML
Cannabigerolic acid (CBGA)	e.g.: Sigma-Aldrich; T-032-1ML
Delta-9-Tetrahydrocannabinolic acid (Δ -9-THCA)	e.g.: Sigma-Aldrich; C-154-1ML
Cannabivarin (CBV)	e.g.: Sigma-Aldrich; C-225-1ML
Cannabichromenic Acid (CBCA)	e.g.: Sigma-Aldrich; C-244-1ML
Cannabicitran (CBT)	e.g.: Cayman Chemical, Item 21295
Cannabielsoin (CBE)	e.g.: Cayman Chemical, Item 36661

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Table 5: List of instruments and consumables

Instruments/Materials	Producer/Supplier and order number
HPLC System	e.g.: Agilent; 1260 infinity
HPLC software	e.g.: Agilent, OpenLab Chromatography Data System (CDS)
Analytical balance	e.g.: Mettler Toledo; XPE204
Test weight	e.g.: Kern & Sohn GmbH; 316-05
Pipets	e.g.: ErgoOne; 10-1000 µl
Tips	e.g.: TipOne; S1161-1820 (1250 µl) and S1163-1800 (200 µl)
Column	e.g.: Agilent; Poroshell 120 SB-C18 (2.7 µm, 4.6 x 150 mm)
Ultrasonic bath	e.g.: ASonic Pro 30
Glass pipets	e.g.: KEFO, 7020294 and 7020295
Syringes 5 and 3 ml	e.g.: KEFO, 9410062 and 9410063
Chromafil Filters	e.g.: KEFO, 7658851
Parafilm	e.g.: KEFO, 9170002
Spatulas	e.g.: KEFO, 9220204 and 9220208
Vials	e.g.: Agilent, 5183-2069 or KEFO 11090520
Vial caps	e.g.: Agilent, 5182-0717 or KEFO 4008218
Cleaning agent for Dishwasher	e.g.: KEFO; 9192425 and 9192446

6. PREPARATION OF REAGENTS, SOLUTIONS, QUALITY CONTROL SAMPLES AND SAMPLES

6.1 Required solutions

Table 6: Preparation of required solution

Solution	Preparation
MPA (0.1 % formic acid)	<u>Preparation:</u> Add 2.5 ml of formic acid to 2.5 l of MQ water and mix the solution by shaking. <u>Stability:</u> 3 days at RT (confirmed during validation).
MPB (0.1 % formic acid + 1.0% MQ water in ACN)	<u>Preparation:</u> Add 2.5 ml of formic acid and 25 ml of MQ water to 2.5 L ACN (originally packed) and mix the solution by shaking. It is recommended that the MPB is prepared one day in advance. <u>Stability:</u> 2 weeks at RT (confirmed with stability experiments, see development report DR-005).
Sample dilution buffer (0.1 % formic acid in MeOH)	<u>Preparation:</u> Add 2.5 ml of formic acid to 2.5 l of MeOH (originally packed) and mix the solution by shaking. <u>Stability:</u> 2 weeks at RT (confirmed during validation).
Column storage solution (70 % ACN)	<u>Preparation:</u> In a volumetric flask measure 700 ml of ACN and fill to the 1000 ml mark with MQ water. Mix by shaking. <u>Stability:</u> 6 months at RT.

Note: If larger or smaller volumes of the solutions are required the quantities in the instructions may be adapted proportionally.

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6.2 Preparation of quality control samples

Quality control samples must be used in every analysis and their results must be tracked in control charts. Based on quality control samples the validity of each analysis is confirmed - as described in SOP-LAB-001.

The quality control samples are used for:

Composition of sample dilution buffer

The blank injections are used to confirm that no cannabinoids are present in the sample dilution buffer(s) and cannabinoids detected can be contributed only to the samples.

Sensitivity of the HPLC system

Sensitivity of HPLC system is tracked with the LOQ solution, which is injected at the beginning of each analysis.

Peak identification and RT tracking (together with the CRM STD)

In any case when the peak identification in the sample is questionable, QCS sample for identification purpose is used, together with CRM STD and REF STD. Sample is overlapped with the QCS sample to assure that the retention time of the unknown sample peak is the same as the retention time of the known peak in the QCS sample.

Conformation of uniformity of chromatographic conditions

Every 15 injections and at least at the end of each sequence the QCS is re-injected to confirm the suitable chromatographic conditions (same RT times of the cannabinoids, same peak shapes, same resolution). The conformation can be confirmed by overlapping the QCS sample.

Resolution tracking

Critical resolution between the CBD and CBG is monitored in REF STD through the resolution tracking in control chart. When adequate resolution between CBD and CBG cannot be achieved, the new column must be used. The column with poor resolution must be restored by vendors recommendations before re-use.

6.2.1 Blank

The same blank is used for all procedures.

The blank sample contains sample dilution buffer and it is used to define a baseline and thus enable the analyst to distinguish sample signals from system signals occurring due to the chromatographic conditions and buffer components. It is crucial that the same dilution buffer as blank is used.

The blank samples are used at the beginning of the sequence, where more than one can be injected, and can be re-injected anywhere in the sequence if necessary - 1 blank should be injected after each of the more complex sample (concentrated, complex matrices etc.). If more than one sample dilution buffer is used for the preparation of the samples in one analysis, all the sample dilution must be proven through the injection and evaluation.

The blank sample is stable up to 1 week in the autosampler (confirmed during validation).

6.2.2 LOQ solution

The same LOQ solution is used for all procedures except for the conformation of Δ -9-THC and Δ -9-THCA.

LOQ solution is diluted CBD crystal, with a final target concentration of 0.3 µg/ml. The solution is prepared in a bulk and aliquoted into 60 vials, each containing 500 µl of the LOQ solution. LOQ solution is used for the system sensitivity evaluation.

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Preparation:

Weight 100 mg of CBD Crystal (purity $\geq 99\%$) in 100 ml of sample dilution buffer. Next use two-steps dilution. First dilute 500 μ l of prepared CBD crystal in 100 ml of sample dilution buffer. In the next step mix 610 μ l of first step dilution in 10 ml of sample dilution buffer. Prepare in triplicates to obtain 3 x 10 ml solution of 0,3 μ g/ml. Mix all the final dilutions to get 30 ml of LOQ solution and aliquot it into 60 vials - each contain 500 μ l LOQ solution. Store in the freezer at -20°C . All the dilutions must be prepared gravimetrically.

Stability:

LOQ solution is stable at least 7 months at -20°C (confirmed and evaluated with stability experiments, see development report DR-001).

Stability in the autosampler: 1 week at $2-8^{\circ}\text{C}$ (confirmed during validation).

6.2.3 QCS and QCS 10xR

The same QCS solution is used for all procedures except for the conformation of Δ -9-THC and Δ -9-THCA. QCS 10xR solution is used only for the procedure MET-LAB-001/01.

QCS is in advance prepared sample which contains all 16 cannabinoids with unknown concentrations. The QCS is used to assess the repeatability of the measurements. The 10-time dilution of QCS (marked as QCS 10xR) is used to assess the concentrations from 3.00 to 30.0 % for the procedure SOP-MET-001-01.

Preparation:

First, the material from which the QCS is made must be selected by the LAB/MAN and must contain:

- all 16 cannabinoids with different concentrations
- at least 2 cannabinoids with concentrations higher than 3.00 and lower than 30 %, to track the repeatability of the higher concentrations of cannabinoids

QCS:

Prepare the QCS following the sample preparation protocol described in Chapter 6.3 (weight 80-120 mg of selected materials into 100 ml volumetric flask). Aliquot the sample - make 60 vials with 1 ml of solution and stored them at -20°C . Each vial must be appropriately marked (as QCS.XX).

QCS 10xR:

Weight 10 ml of solution from previous step (QCS) in 100 ml of measuring flask and fill it to the 100 ml mark with the sample dilution buffer. Weight the final solution. Mix well and fill 1 ml of QCS.10xR into 60 vials. Each vial must be appropriately marked (as QCS 10xR.XX).

Each preparation of the QCS and QCS 10xR must be marked with the chronological number (QCS.01, QCS.02, QCS.03...

Stability:

QCS and QCS 10xR are stable:

- up to 1 week in the autosampler ($2-8^{\circ}\text{C}$) - confirmed during validation
- up to 5 months at -20°C - proved by the evaluation of QCS.01

6.2.4 REF STD 40xR

REF STD 40xR solution is used only for the procedure MET-LAB-001/1.

REF STD 40xR is in advanced prepared dilution of the sample, which was prepared from the mixture of CBD and CBG isolates with high confirmed purity - above 98 % for CBG and above 99 % for CBD. The preparation of REF STD 40xR has to be prepared gravimetrically and the final concentrations of CBD and CBG has to be calculated. After the preparation the calculated concentrations must be checked and entered in the new control chart, prepared by MAN/LAB. In control chart the accuracy for CBD and CBG and resolution between CBD and CBG are tracked. See SOP-LAB-001 for more details.

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Preparation:

Step 1: Weight 100 mg of CBD and 100 mg of CBG into 100 ml of MeOH (the same preparation as for normal samples). After the extraction perform the step 2 described below.

Step 2: Sample from step 1 must be diluted by a factor of 40 to fit into linear range of the method. Weight 2.5 ml of the sample from the step 1 into 100 ml of volumetric flask and fill to the 100 ml with the dilution buffer. Prepare gravimetrically.

Each preparation of the REF STD 40xR has to be marked with the chronological number (REF STD 40xR.01, REF STD 40xR.02, REF STD 40xR.03, ...)

Stability:

REF STD 40xR is stable:

- up to 1 week in the autosampler (2-8°C) - confirmed during validation
- up to 10 months at -20°C - proved by the evaluation of REF STD.01 40xR

6.2.5 REF STD 4xR

REF STD 4xR solution is used only for the procedure MET-LAB-001/02.

REF STD 4xR is in advanced prepared dilution of the sample, which was prepared from the mixture of CBD and CBG isolates with high confirmed purity - above 98 % for CBG and above 99 % for CBD. The preparation of REF STD 4xR must be prepared gravimetrically and the final concentrations of CBD and CBG must be calculated. After the preparation the calculated concentrations must be checked and entered in the new control chart, prepared by MAN/LAB. See SOP-LAB-001 for more details.

Preparation:

Step 1: Weight 100 mg of CBD and 100 mg of CBG into 100 ml of MeOH (the same preparation as for normal samples). After the extraction perform the step 2 described below.

Step 2: Sample from step 1 must be diluted by a factor of 4 to fit into linear range of the method. Weight 25 ml of the sample from the step 1 into 100 ml of volumetric flask and fill to the 100 ml with the dilution buffer. Prepare gravimetrically.

Each preparation of the REF STD 4xR must be marked with the chronological number (REF STD 4xR.01, REF STD 4xR.02, REF STD 4xR.03, ...)

Stability:

REF STD 4xR is stable:

- up to 1 week in the autosampler (2-8°C) - confirmed during validation
- up to 10 months at -20°C - proved by the evaluation of REF STD.01 40xR

6.2.6 CRM STD

The same CRM solution is used for all procedures.

CRM STD is in advanced prepared dilution of the combined 15 certified reference standards - CBDV, CBDA, CBV, CBGA, CBG, CBD, THCV, CBN, Δ-9-THC, Δ-8-THC, CBL, CBC, Δ-9-THCA, CBCA and CBT thus the CRM STD is containing the accurate cannabinoids concentrations, which are traceable to the International System of units (SI). See SOP-LAB-001 for more details.

Preparation:

Prepare 500 µl of each CRM into 20 ml volumetric flask and fill to the 20 ml mark with the sample dilution buffer. Weight each CRM separately and weight the final solution (prepare all of the steps gravimetrically).

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Aliquot 500 µl of solution into vials and store at - 20°C. After the preparation the calculated concentrations must be checked and entered in the new control chart, prepared by MAN/LAB. See SOP-LAB-001 for more details.

Each preparation of the CRM STD has to be marked with the chronological number (CRM STD.01, CRM STD.02, CRM STD.03, ...)

Stability:

CRM STD is stable:

- up to 1 week in the autosampler (2-8°C) - confirmed during validation
- up to 6 months at -20°C - proved by the evaluation of CRM STD.01

6.2.7 CRM STD.XX_9THC_9THCA

CRM STD.XX_9THC_9THCA is used for the procedure MET-LAB-001/03

CRM STD.XX_9THC_9THCA is in advanced prepared dilution of the cannabinoids Δ-9-THC and Δ-9-THCA from CRM, thus contains the accurate concentration of Δ-9-THC and Δ-9-THCA, which is traceable to the SI.

Preparation:

Prepare 50 µl of each CRM (Δ-9-THC and Δ-9-THCA) into 20 ml volumetric flask and fill to the 20 ml mark with the sample dilution buffer. Weight each CRM separately and weight the final solution (prepare all the steps gravimetrically). Aliquot 800 µl of solution into vials and store at - 20°C. After the preparation the calculated concentrations must be checked and entered in the new control chart, prepared by MAN/LAB. See SOP-LAB-001 for more details.

Each preparation of the CRM STD must be marked with the chronological number (CRM STD.01_9THC_9THCA, CRM STD.02_9THC_9THCA, CRM STD.03_9THC_9THCA, ...)

Stability:

CRM STD_9THC_9THCA is stable:

- up to 1 week in the autosampler (2-8°C) - confirmed during validation
- up to 6 months at -20°C - proved by the evaluation of CRM STD.01

6.3 Sample preparation

Preparation of undiluted and non-concentrated sample:

1. Weight 100 mg of sample in 100 ml volumetric flask or 10 mg of sample in 10 ml volumetric flask on analytical balance.
2. Dilute the sample in 100- or 10- mL sample dilution buffer using the mark on the volumetric flask (bottom of a meniscus).
3. Close the measuring flask tightly and start the extraction process. The prefer option is ultrasonic-assisted extraction but it is not optimal for all samples. Extraction without the ultrasound must be performed if the portion of the sample is not diluted in methanol.
4. When performing the ultrasound-assisted extraction run the ultrasonic bath for 15 min at ambient temperature and mix the sample every 5 min by shaking the volumetric flask. When extraction is done without ultrasound shake the samples more aggressively and turn the flask upside down until all the sample is completely dissolved in the methanol. Extraction should last for at least for 15 min and shaking and rotating of the flask should be applied every 2-4 min.
5. After the extraction filter the portion of the sample through 0.45 µm filter (e.g.: Chromafil Xtra H-PTFE).

The concentration of each cannabinoid must be in the concentration range of the procedure used. The range of each procedure was evaluated and confirmed by validation of analytical method/procedure and is

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summarized in the validation report. Depending on the sample composition and procedure used, the sample may need to be further diluted with sample dilution buffer.

Preparation of 10 times concentrated sample:

- Suitable for all matrices / sample types.
- The initial weight of the weighted sample is increased by 10 times
- The sample is prepared the same as non-concentrated sample

The concentration of each cannabinoid must be in the concentration range of the method from 0.3 to 30.0 ug/ml. Depending on the sample preparation and sample composition, the sample may need to be further diluted with sample dilution buffer.

Preparation of 100 times concentrated sample:

- Suitable only for liquids and viscous liquids, oils and plant materials
- The initial weight of the weighted sample is increased by 100 times
- The sample is prepared the same as non-concentrated sample

The 100 times concentrated sample can be prepared for other matrices to confirm the concentration from 0.0003 to 0.003 for only certain cannabinoids if the suitability of the matrix is confirmed by comparing the chromatograms of 10x and 100x concentrated samples. Any potential matrix interferences must be evaluated to confirm the suitability to report specific cannabinoid below 0.003 %. The results between 10x and 100x concentrated sample must be comparable (results within the expanded measurement uncertainty).

6.3.1 Sample types with different sample preparation protocol

Some sample types require the adaptation of the sample preparation protocol. The adaptation was confirmed during validation study - see validation report RP-VP-011 - Analytical method validation report for method MET-003.

Preparation of gummy materials:

Due to the poor suitability of the gummy materials the following sample preparation protocol is required:

1. Weight 100 - 1000 mg of sample in a volumetric flask on analytical balance.
2. Add 10 ml of MQ water and mix thoroughly. Put the sample in the water bath at 40-70°C until the sample is completely dissolved in the MQ water.
3. Add the sample dilution buffer to the 100 ml mark on the volumetric flask.
4. Close the measuring flask tightly and start the extraction process.
5. Run the ultrasonic bath for 15 min at ambient temperature and mix the sample every 5 min by shaking the volumetric flask.
6. After the extraction filter the portion of the sample through 0.45 µm filter.

Prepare the diluted sample if needed as described in procedures in Chapter 7, 8 and 9. Depending on the sample preparation and sample composition, the sample may need to be further diluted with sample dilution buffer.

Preparation of balms:

Due to the poor suitability of the balm the following sample preparation protocol is required:

1. Weight 100 - 1000 mg of sample in a volumetric flask on analytical balance.
2. Add 10 ml of MCT oil and mix thoroughly. Put the sample in the water bath at 40-70°C until the sample is completely dissolved in the MCT oil.
3. Add the sample dilution buffer to the 100 ml mark on the volumetric flask.
4. Close the measuring flask tightly and start the extraction process.
5. Run the ultrasonic bath for 15 min at ambient temperature and mix the sample every 5 min by shaking the volumetric flask.
6. After the extraction filter the portion of the sample through 0.45 µm filter (e.g. Chromafil Xtra H-PTFE).

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Prepare the diluted sample if needed as described in procedures in Chapter 7, 8 and 9. Depending on the sample preparation and sample composition, the sample may need to be further diluted with sample dilution buffer.

Stability: 1 week in autosampler (2-8°C) or 1 week in a freezer (-20°C) - confirmed during validation. If the conditions are not met the sample preparation must be repeated (in cases of electrical outages).

6.4 Recommended conditions and storage

Before column installation and after column removal

The HPLC system should be flushed for at least 20 minutes with solution for column storage (70 % ACN) at 1.2 mL/min.

New column

Before using a new column first flush the column with at least 5 column volumes of 70 % ACN (at least 12.5 mL, e.g.: for a flow rate of 1.2 ml/min it would take at least 10 minutes) to remove potential residual impurities. Follow the vendors recommendation and gradually increase the flow to 1.2 ml/min.

Next equilibrate the column with the initial mobile phase gradient for 30 minutes with a flow rate of 1.2 mL/min. Then twice inject 10 µl of 100% MeOH (20 min run with the gradient for sample analysis).

Already used column

Flush an already used column with the initial mobile phase gradient for 30 minutes with a flow rate of 1.2 mL/min. Follow the vendors recommendation and gradually increase the flow to 1.2 ml/min.

Column storage

The column must be stored in ACN/MQ water mixture (without HFO), 70% ACN (v/v) is recommended. After each sequence equilibrate the column with long term column storage solution (30 minutes with a flow rate of 1.2 mL/min). Column is stored at room temperature, installed on the system or in the provided box.

Conditioning of the HPLC system

Use initial mobile phase gradient and gradually increase the flow on the HPLC system from 0.1 to 1.2 mL/min. Monitor the pressure on the system and when is stable wait for another 15 min before starting the sequence.

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7. PROCEDURE MET-LAB-001/01

Use this procedure only on system HPLC1.

7.1 Preparation of certified reference standard material solutions for calibration

Concentration of cannabinoids in samples are determined using calibration curve from the known concentration of specific cannabinoid - dilutions from CRMs. Calibration of the method was performed during validation and must be repeated at least once a year, if all the acceptance criteria are met during routine testing of the method (see SOP-LAB-001).

Preparation:

Prepare CAL9N and CAL3A dilutions with final concentration of 60 µg/ml (see Table 6 and 7). Dilutions must be prepared gravimetrically.

Table 7: Reference standards dilution for method calibration (CAL9N)

CRM/Material	Volume [µl]	Estimate final C [µg/ml]
CBDV	100.0	60
THCV	100.0	60
CBG	100.0	60
CBD	100.0	60
Δ-9-THC	100.0	60
CBN	100.0	60
CBC	100.0	60
Δ-8-THC	100.0	60
CBL	100.0	60
Methanol	767.0	/

Table 8: Reference standards dilution for method calibration (CAL3A)

CRM/Material	Volume [µl]	Estimate final C [µg/ml]
CBDA	72.0	60
CBGA	72.0	60
Δ-9-THCA	72.0	60
Methanol	984.0	/

Next use each of prepared dilutions of CAL9N and CAL3A for further dilutions with MeOH to obtain different concentration levels. Prepare one dilution for each standard concentration level, covering the range from 0.3 to 30 µg/ml (see Table 8 and 9). Prepare all dilutions gravimetrically.

Table 9: Reference standard dilution and injection volumes for Calibration (CAL7N)

Sample name	Start C [µg/ml]	Sample V [µl]	Solvent V (MeOH) [µl]	Final sample C [µg/ml]
CAL9N_30*	60.0	500	500	30.0
CAL9N_15*	30.0	500	500	15.0
CAL9N_7.5*	15.0	500	500	7.5
CAL9N_3.7*	7.5	500	500	3.7
CAL9N_1.8*	3.7	500	500	1.8
CAL9N_0.9*	1.8	500	500	0.9
CAL9N_0.3	0.9	200	400	0.3

Abbreviations: V = volume, C = concentration *Used also for further dilution

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Table 10: Reference standard dilution and injection volumes for Calibration (CAL3A)

Sample name	Start C [µg/ml]	Sample V [µl]	Solvent V (MeOH) [µl]	Final sample C [µg/ml]
CAL3A_30*	60.0	500	500	30.0
CAL3A_15*	30.0	500	500	15.0
CAL3A_7.5*	15.0	500	500	7.5
CAL3A_3.7*	7.5	500	500	3.7
CAL3A_1.8*	3.7	500	500	1.8
CAL3A_0.9*	1.8	500	500	0.9
CAL3A_0.3	0.9	200	400	0.3

Abbreviations: V = volume, C = concentration *Used also for further dilution

First calculate the accurate concentration of each cannabinoid in the CAL9N and CAL3A mixtures. Next calculate the accurate concentration for each cannabinoid for each level. Enter calculated values into required fields in the OpenLAB software - defined in the Levels 1 to 7 in Peaks/Groups category (see the manual for advanced use of the OpenLAB software).

Stability:

7 months at -20°C (confirmed during the development, see development report DR-004 - Primerjava certificiranih referenčnih materialov).

7.2 Additional remarks regarding the sample preparation

Preparation of diluted sample (for non-concentrated samples):

The dilutions of the initial samples are prepared accordantly to the following:

- Only undiluted sample is prepared if the concentration for all cannabinoids is in the range from 0 to 30.0 µg/ml
- 10x dilution is prepared if the concentrations for all cannabinoids are in the range from 0 to 300 µg/ml
- 20x dilution is prepared if the concentrations for all cannabinoids are in the range from 0 to 600 µg/ml
- 30x dilution is prepared if the concentrations for all cannabinoids are in the range from 0 to 900 µg/ml
- 100x dilution is prepared if the concentrations for all cannabinoids are in the range from 0 to 1000 µg/ml

7.3 HPLC parameters and analysis set-up

7.3.1 HPLC system

HPLC parameters and conditions are described in a table below.

Table 11: HPLC parameters and conditions

Parameter	Condition
Flow rate	1.2 mL/min
Column temperature	28°C OpenLab settings: Temperature delta: 2°C
Injection volume	10.0 µL
Run Time	20 minutes
Auto-sampler temperature	5°C OpenLab settings: Lower limit: 4°C / Upper limit: 7°C
Detection	237 nm
Step for the UV detector	OpenLab settings: > 0.1 min (2 s response time) (5 Hz) (corresponds to a step of 0.2 s)
DAD spectrum (optional)	190.0 - 400.0 nm, step 2.0 nm

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7.3.2 Gradient for sample analysis

Table 12: Gradient for the sample analysis

Time [min]	Mobile phase B [%]
0.0	70
6.00	70
12.00	77
17.00	77
17.20	70

7.3.3 Sequence set- up

Table 13: Example of sequence of injections up to 43 samples

Sample name	No. of injections
Blank	1-3
LOQ	1
QCS 10x	1
QCS	1
REF STD 40xR	1
CRM STD	1
Sample 1	1
Sample...	1
Sample 13	1
QCS	1
Sample 14	1
Sample...	1
Sample 28	1
QCS	1
Sample 29	1
Sample...	1
Sample 43	1
QCS	1

7.4 Evaluation and result reporting

7.4.1 Evaluation

Determine the peak area of each peak in the chromatogram, which are not present in the blank sample. Integration of the peaks that are present in the blank sample is not mandatory. Use correct baseline integration (see Chapter 10).

7.4.2 Result reporting

All the cannabinoids must be in the concentration range of the method; thus, the following rules must be followed:

For 100 mg of sample per 100 ml of sample dilution buffer:

1. If the concentrations of all cannabinoids are from 0.03 to 3.00 % w/w only the results for undiluted sample are considered. If there are any of the dilutions recorded, they do not have to be integrated or taken into consideration.
2. If the concentration of any cannabinoid exceeds the 3.00 % w/w, for that cannabinoid the reported result is:
 - from 10x dilution: if cannabinoid is in the range from 3.00 % w/w to 30.0 % w/w
 - from 20x dilution: if cannabinoid is in the range from equal or greater than 30.0% w/w and 60.0 % w/w
 - from 30x dilution: if cannabinoid is in the range from equal or greater than 90. to 90.0 % w/w
 - from 100x dilution: if cannabinoid is in the range from equal or greater than 90 % w/w

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If the sample is concentrated the different rules for reporting apply:

- the LOQ value (in % w/w) for reported result is lower due to the more sample in the solution
- the range of the analytical method is the same (0.3 to 30.0 ug/ml), which means that the reporting ranges for % are lower. See DR-008 - Vpliv koncentriranja vzorcev na določitev vsebnosti kanabinoidov and the example below for more details.

For example, if the sample is 50 times concentrated, the LOQ value is 50 times lower (0.0006 % w/w) and the highest reported value becomes 0.06 % w/w.

Report all the result values $\geq$ LOQ to the number of decimal places accordantly to the calculated Measurement uncertainty decimal places. Report the concentrations below LOQ as "<LOQ. Report the non-existing or missing peaks "<LOQ" or "ND." The reporting of "ND" is possible – for cannabnoids that are not present or below the detection limit of the procedure. ND limits are explained in DR-011 - Meja detekcije za metodo MET-001.

7.5 Acceptance criteria (SST)

Blank

No interfering peak detection in the blank with a signal height $\geq$ LOQ signal height, in the RT windows of the known cannabinoids (present in the QCS sample).

Sensitivity

For each analysis the LOQ is evaluated using the S/N ratio. Acceptance criterion: $S/N \geq 10$. The S/N ratio is evaluated using the following formula:

$$S/N = 2H_{CBD}/h$$

H is the CBD signal height; h is the noise level. Signal height is evaluated by the OpenLAB software and can be displayed in the report. For noise level determination chromatography data system use the blank injection before LOQ injection. The calculation is based on the noise over a range of at least 5 times the peak width at half peak height. In most cases automatic approach is suitable for the noise region determination, but manual region can also be adapted.

Cannabinoids concentrations in the QCS, REF STD 40xR and CRM

The result for each cannabinoid is entered in the control chart and based on the trend or concentration the acceptance criteria are established as described in SOP-LAB-001.

Resolution

Resolution is evaluated on quality control sample REF STD between CBD and CBG and tracked in corresponding control charts. The acceptance criterion is established as described in SOP-LAB-001.

Accuracy

Accuracy of the analytical method must be checked every month with the CRM. It is important, that each time the different cannabinoid is evaluated in that way that every 12 months 12 cannabinoids are evaluated. For each cannabinoid the following concentrations must be prepared and evaluated: 30.0, 15.0, 7.5, 3.7, 1.8 and 0.9 µg/ml. All dilutions must be prepared gravimetrically. Follow the SOP-LAB-001 for more information.

Procedure re-calibration

Procedure re-calibration is required each year. It is recommended that the fresh, not open certified reference standards are used. Each calibration must be compared with the previous calibration and evaluated in the development report (DR-XXX).

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7.6 Data documentation and Control Charts

Analysis must be documented. All documents must be either e-signed or signed as hard copies. The results must be checked and signed. The responsibilities of personnel are described in SOP-LAB-009 - Delovne naloge, odgovornosti in pooblastila.

Control chart (i.e., in the form of an Excel table) relating the method should be stored in the corresponding directory, which is backed up to the server daily.

8. PROCEDURE MET-LAB-001/02

Use this procedure only on system HPLC2.

8.1 Preparation of certified reference standard material solutions for calibration

Concentration of cannabinoids in samples are determined using calibration curve from the known concentration of specific cannabinoid - dilutions from certificated reference standards. Calibration of the method was performed during validation but must be repeated at least once a year, if all the acceptance criteria are met during routine testing of accuracy of the method (see SOP-LAB-001).

Preparation:

Prepare each calibration standard individually as described in the following tables. Prepare one solution for each calibration level. Prepare all dilutions gravimetrically. If larger or smaller volumes of the solutions are required the quantities in the instructions may be adapted proportionally.

Table 14: Calibration points for procedure MET-LAB-001/2

Sample ID	Concentration [µg/ml]	Prepared from
Cal 300	300	CRM
Cal 200	200	CRM
Cal 100	100	Cal 200
Cal 50.0	50.0	Cal 100
Cal 25.0	25.0	Cal 50.0
Cal 12.5	12.5	Cal 25.0
Cal 1.25	1.25	Cal 12.5
Cal 0.30	0.30	Cal 1.25

Table 15: Preparation of calibration points

Sample ID	Start concentration [µg/ml]	Final concentration [µg/ml]	Sample V [µl]	MeOH V [µl]
Cal 300	1000	300	60	140
Cal 200*	1000	200	200	800
Cal 100*	200	100	500	500
Cal 50.0*	100	50.0	500	500
Cal 25.0*	50.0	25.0	500	500
Cal 12.5*	25.0	12.5	500	500
Cal 1.25*	12.5	1.25	100	900
Cal 0.30	1.25	0.30	240	760

* Used for further dilutions

First calculate the accurate concentration of each cannabinoid in final mixtures, to get the accurate concentration for each cannabinoid for each level. Enter calculated values into required fields in the OpenLAB software - defined in the Levels 1 to 8 in Peaks/Groups category. Follow the NZD-LAB-010 - Navodila za uporabo programske opreme OpenLAB.

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Stability:

7 months at -20°C (confirmed during the development, see development report DR-004 - Primerjava certificiranih referenčnih materialov).

8.2 Additional remarks regarding the sample preparation

The concentration of each cannabinoid must be in the concentration range of the method from 0.3 to 300 µg/ml for CBDV, CBDA, CBGA, THCV, CBN, Δ-9-THC, CBC, Δ-9-THCA, Δ-8-THC, CBL, CBE, CBV, CBCA; for CBD and CBG the concentration range of the method is from 0.3 to 800 µg/ml. The range was evaluated and confirmed by validation of analytical method and is summarized in the validation report.

Depending on the concentration of any of the cannabinoid, sometimes one step dilution is required.

8.3 HPLC parameters and analysis set-up

8.3.1 HPLC system

HPLC parameters and conditions are described in the table below.

Table 16: HPLC parameters and conditions

Parameter	Condition
Flow rate	1.2 mL/min
Column temperature	40°C OpenLab settings: Temperature delta: 0.8°C
Injection volume	10.0 µL
Run Time	12.2 minutes
Auto-sampler temperature	4°C
Detection	276 nm
Step for the UV detector	OpenLab settings: > 0.1 min (2 s response time) (5 Hz) (corresponds to a step of 0.2 s)
DAD spectrum (optional)	190.0 - 400.0 nm, step 2.0 nm

8.3.2 Gradient for sample analysis

Table 17: Gradient for the sample analysis

Time [min]	Mobile phase B [%]
0.0	72
3.0	72
4.2	74
4.3	82
10.1	82
10.2	72

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8.3.3 Sequence set- up

Table 18: Example of sequence of injections up to 28 samples

Sample name	No. of injections
Blank	1-3
LOQ	1
QCS	1
REF STD 4xR	1
CRM STD	1
Sample 1	1
Sample...	1
Sample 13	1
QCS	1
Sample 14	1
Sample...	1
Sample 28	1
QCS	1

Note: If required, more samples can be analyzed in one sequence

8.4 Evaluation and result reporting

8.4.1 Evaluation

Determine the peak area of each peak in the chromatogram, which are not present in the blank sample. Integration of the peaks that are present in the blank sample is not mandatory. Use correct baseline integration (see Chapter 10).

8.4.2 Result reporting

All the cannabinoids must be in the concentration range of the method; thus, the following rules must be followed:

1. If the concentrations of all cannabinoids are from 0.03 to 30.0 % w/w only the results for undiluted sample are considered. If the concentrations for CBD or CBG are from 0.03 to 80.0 % w/w also the results for undiluted sample are considered.

If the sample is concentrated the different rules for reporting apply:

- the LOQ value (in % w/w) for reported result is lower due to the more sample in the solution
- the range of the analytical method is the same (0.3 to 30.0 ug/ml), which means that the reporting ranges for % are lower. See DR-008 - Vpliv koncentriranja vzorcev na določitev vsebnosti kanabinoidov and the example below for more details.

For example, if the sample is 50 times concentrated, the LOQ value is 50 times lower (0.0006 % w/w) and the highest reported value become 0.06 % w/w.

Report all the result values $\geq$ LOQ to the number of decimal places accordantly to the calculated Measurement uncertainty decimal places. Report the concentrations below LOQ as "<LOQ. Report the non-existing or missing peaks "<LOQ."

8.5 Acceptance criteria (SST)

Blank

No interfering peak detection in the blank with a signal height $\geq$ LOQ signal height, in the RT windows of the known cannabinoids (present in the QCS sample).

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Sensitivity

For each analysis the LOQ is evaluated using the S/N ratio. Acceptance criterion: $S/N \geq 10$. The S/N ratio is evaluated using the following formula:

$$S/N = 2H_{CBD}/h$$

H is the CBD signal height; h is the noise level. Signal height is evaluated by the OpenLAB software and can be displayed in the report. For noise level determination chromatography data system use the blank injection before LOQ injection. The calculation is based on the noise over a range of at least 5 times the peak width at half peak height. In most cases automatic approach is suitable for the noise region determination, but manual region can also be adapted.

Cannabinoids concentrations in the QCS, REF STD 4xR and CRM

The result for each cannabinoid is entered in the control chart and based on the trend or concentration the acceptance criteria are established as described in SOP-LAB-001.

Resolution

Resolution is evaluated on quality control sample REF STD between CBD and CBG and tracked in corresponding control charts. The acceptance criterion is established as described in SOP-LAB-001.

Accuracy

Accuracy of the analytical method must be checked every month with the CRM. It is important, that each time the different cannabinoid is evaluated in that way that every 12 months or 12 cannabinoids are evaluated. For CBD and CBG the following concentrations must be prepared and evaluated: 800.0, 600.0, 300.0, 30.0, 3.0 and 0.30 µg/ml and for other cannabinoids the following concentrations must be evaluated: 300.0, 150.0, 100.0, 50.0, 3.0 and 0.30 µg/ml. All dilutions must be prepared gravimetrically. Follow the SOP-LAB-001 for more information.

Procedure re-calibration

Procedure re-calibration is required each year. It is recommended that the fresh, not open certified reference standards are used. Each calibration must be compared with the previous calibration and evaluated in the development report (DR-XXX).

8.6 Data documentation and Control Charts

Analysis must be documented. All documents must be either e-signed or signed as hard copies. The results must be checked and signed. The responsibilities of personnel are described in SOP-LAB-009 - Delovne naloge, odgovornosti in pooblastila.

Control chart (i.e., in the form of an Excel table) relating the method should be stored in the corresponding directory, which is backed up to the server daily.

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9. PROCEDURE MET-LAB-001/03

Use this procedure only on system HPLC1.

When the Δ -9-THC and/or Δ -9-THCA in the sample could not be determined as seen in the Figure 1, use this procedure, which enables the quantification of Δ -9-THC and/or Δ -9-THCA in the sample as seen on Figure 2.

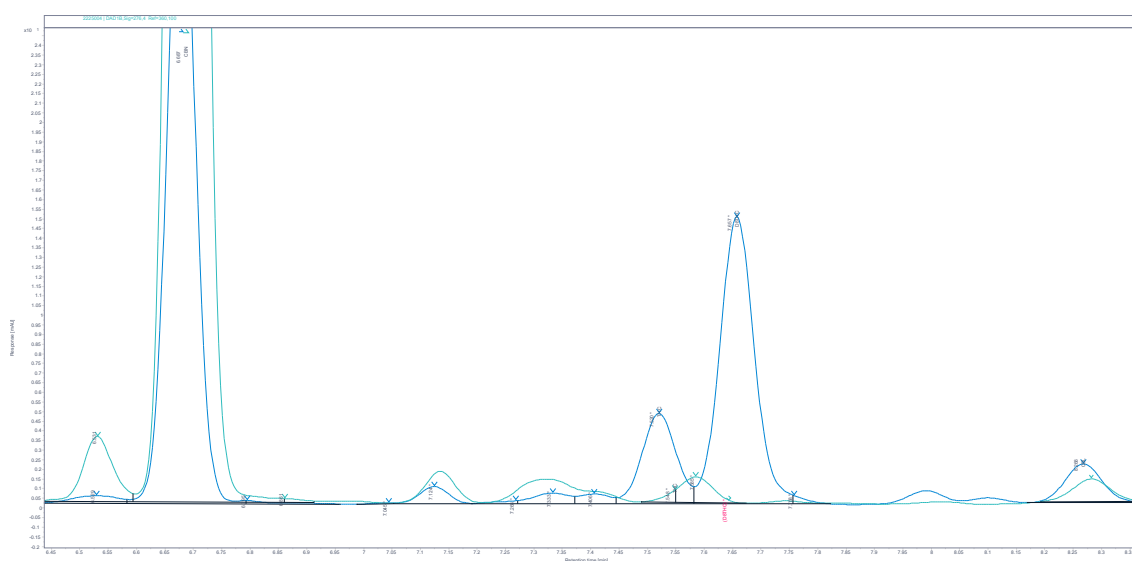


Figure 1: Procedure MET-LAB-001/01: Chromatogram of the sample ID: 2225004 overlaid with the chromatogram of QCS; zoom-in around Δ -9-THC and Δ -8-THC retention times (sample in green, QCS in blue).

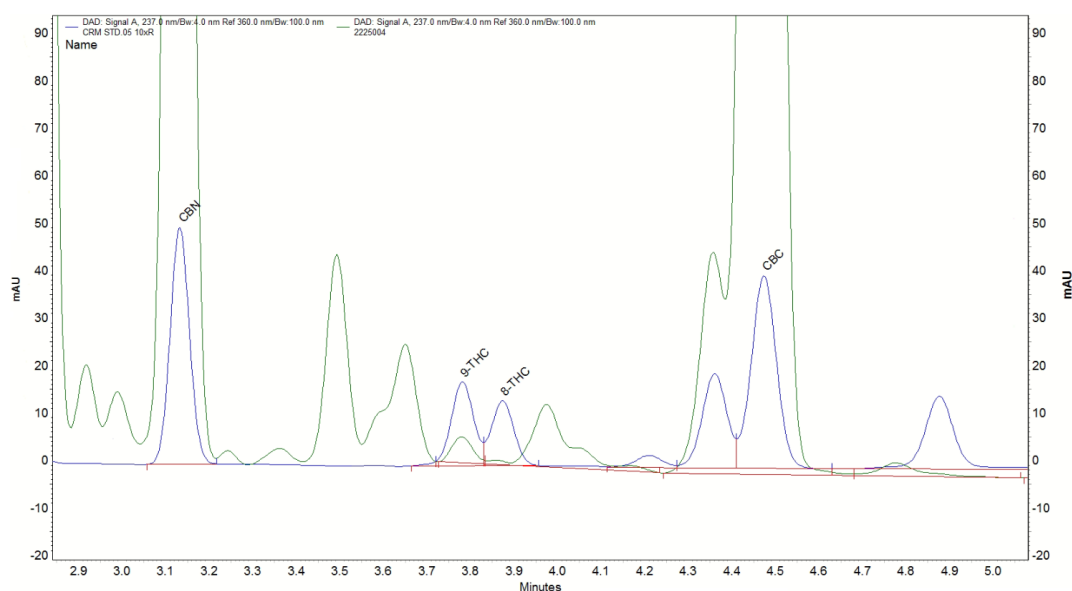


Figure 2: Procedure MET-LAB-001/03: Chromatogram of the sample ID: 2225004 overlaid with the chromatogram of CRM; zoom-in around Δ -9-THC and Δ -8-THC retention times (sample in green, CRM in blue).

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9.1 Preparation of certified reference standard material solutions for calibration

Concentration of Δ -9-THC and Δ -9-THCA in samples are determined using calibration curves prepared from CRMs. Calibration of the procedure was performed during validation and must be repeated at least once a year, if all the acceptance criteria are met during routine testing of the procedure (see SOP-LAB-001).

Preparation:

Prepare Δ -9-THC and Δ -9-THCA dilutions separately with different concentration. First prepare the concentration of 100 μ g/ml, which is used for further dilutions for each standard concentration level, covering the range from 0.3 to 30 μ g/ml (see tables 19 and 20). Prepare all dilutions gravimetrically.

Table 19: Reference standard dilution and injection volumes for Calibration (Δ -9-THC)

Sample name	Start C [μ g/ml]	Sample V [μ l]	Solvent V (MeOH) [μ l]	Final sample C [μ g/ml]
Δ -9-THC _100*	1000.0	100	900	100.0
Δ -9-THC _30*	100.0	300	700	30.0
Δ -9-THC _15*	30.0	500	500	15.0
Δ -9-THC _7.5*	15.0	500	500	7.5
Δ -9-THC _3.7*	7.5	500	500	3.7
Δ -9-THC _1.8*	3.7	500	500	1.8
Δ -9-THC _0.9*	1.8	500	500	0.9
Δ -9-THC _0.3	0.9	200	400	0.3

Abbreviations: V = volume, C = concentration *Used also for further dilution

Table 20: Reference standard dilution and injection volumes for Calibration (Δ -9-THCA)

Sample name	Start C [μ g/ml]	Sample V [μ l]	Solvent V (MeOH) [μ l]	Final sample C [μ g/ml]
Δ -9-THCA _100*	1000.0	100	900	100.0
Δ -9-THCA _30*	100.0	300	700	30.0
Δ -9-THCA _15*	30.0	500	500	15.0
Δ -9-THCA _7.5*	15.0	500	500	7.5
Δ -9-THCA _3.7*	7.5	500	500	3.7
Δ -9-THCA _1.8*	3.7	500	500	1.8
Δ -9-THCA _0.9*	1.8	500	500	0.9
Δ -9-THCA _0.3	0.9	200	400	0.3

Abbreviations: V = volume, C = concentration *Used also for further dilution

First calculate the accurate concentration of each cannabinoid in final mixtures, to get the accurate concentration for each cannabinoid for each level. Enter calculated values into required fields in the OpenLAB software - defined in the Levels 1 to 8 in Peaks/Groups category. Follow the NZD-LAB-008 - Navodilo za delo s programsko opremo OpenLAB CDS.

Stability:

7 months at -20°C (confirmed during the development, see development report DR-004 - Primerjava certificiranih referenčnih materialov).

9.2 HPLC parameters and analysis set-up

9.2.1 HPLC system

HPLC parameters and conditions are described in a table below.

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Table 21: HPLC parameters and conditions

Parameter	Condition
Flow rate	1.5 mL/min
Column temperature	40°C OpenLab settings: Temperature delta: 2°C
Injection volume	10.0 µL
Run Time	7 minutes
Auto-sampler temperature	4°C OpenLab settings: Lower limit: 4°C / Upper limit: 7°C
Detection	237 nm
Step for the UV detector	OpenLab settings: > 0.1 min (2 s response time) (5 Hz) (corresponds to a step of 0.2 s)
DAD spectrum (optional)	190.0 - 400.0 nm, step 2.0 nm

9.2.2 Isocratic conditions for sample analysis

Table 22: Isocratic conditions for the sample analysis

Time [min]	Mobile phase B [%]
0.0 – 7.0 min	82

9.2.3 Sequence set- up

Table 23: Example of sequence of injections up to 10 samples

Sample name	No. of injections
Blank	1-3
CRM STD	1
CRM STD_9THC_9THCA	1
Sample 1	1
Sample...	1
Sample 10	1

9.3 Evaluation and result reporting

9.3.1 Evaluation

Determine the peak area of each peak in the chromatogram, which are not present in the blank sample. Integration of the peaks that are present in the blank sample is not mandatory. Use correct baseline integration (see Chapter 10).

9.3.2 Result reporting

The 9-THC and 9-THCA must be in the concentration range of the method, the same rules as for the procedure MET-LAB-001/01 apply:

For 100 mg of sample per 100 ml of sample dilution buffer:

1. If the concentrations of all cannabinoids are from 0.03 to 3.00 % w/w only the results for undiluted sample are considered. If there are any of the dilutions recorded, they do not have to be integrated or taken into consideration.
2. If the concentration of any cannabinoid exceeds the 3.00 % w/w, for that cannabinoid the reported result is:
 - from 10x dilution: if cannabinoid is in the range from 3.00 % w/w to 30.0 % w/w
 - from 20x dilution: if cannabinoid is in the range from equal or greater than 30.0% w/w and 60.0 % w/w
 - from 30x dilution: if cannabinoid is in the range from equal or greater than 90. to 90.0 % w/w
 - from 100x dilution: if cannabinoid is in the range from equal or greater than 90 % w/w

QUANTITATIVE DETERMINATION OF CANNABINOIDS BY REVERSE PHASE CHROMATOGRAPHY

If the sample is concentrated the different rules for reporting apply:

- the LOQ value (in % w/w) for reported result is lower due to the more sample in the solution
- the range of the analytical method is the same (0.3 to 30.0 µg/ml), which means that the reporting ranges for % are lower. See DR-008 - Vpliv koncentriranja vzorcev na določitev vsebnosti kanabinoidov and the example below for more details.

For example, if the sample is 50 times concentrated, the LOQ value is 50 times lower (0.0006 % w/w) and the highest reported value becomes 0.06 % w/w.

Report all the result values $\geq$ LOQ to the number of decimal places accordantly to the calculated Measurement uncertainty decimal places. Report the concentrations below LOQ as "<LOQ. Report the non-existing or missing peaks "<LOQ" or "ND." The reporting of "ND" is possible – when 9-THC or 9-THCA are not present in the sample or its concentration is below the detection limit (LOD) of the procedure. The LOD were tested during the validation of the procedure and are reported in validation report RP-VP-LAB-22-002.

9.4 Acceptance criteria (SST)

Blank

No interfering peak detection in the blank with a signal height $\geq$ LOQ signal height, in the RT windows of the known cannabinoids (present in the QCS sample).

Accuracy

Accuracy of the analytical method must be checked in every analysis with the CRM solutions (two different CRM solutions are used in each analysis).

Procedure re-calibration

Procedure re-calibration is required each year. It is recommended that the fresh, not open certified reference standards are used. Each calibration must be compared with the previous calibration and evaluated in the development report (DR-XXX).

9.5 Data documentation and Control Charts

Analysis must be documented. All documents must be either e-signed or signed as hard copies. The results must be checked and signed. The responsibilities of personnel are described in SOP-LAB-009 - Delovne naloge, odgovornosti in pooblastila.

Control chart (i.e., in the form of an Excel table) relating the method should be stored in the corresponding directory, which is backed up to the server daily.

10. INTEGRATION AND PROCESSING RULES

General instruction:

Integration of chromatograms is performed automatically by using the OpenLAB software, but can be in addition corrected manually. Automatic peak labeling may not be correct due to the peak RT shifting. In that case it must be corrected manually according to the retention times of the peaks in the QCS or CRM.

Peak integration:

Integration must be done at least for all those signals that occur in the sample run and are absent in the blank and are in the retention window of the expected cannabinoids. Artifacts like baseline disturbances or gradient drift must be excluded from the integration (see examples: Figure 3 and 4).

QUANTITATIVE DETERMINATION OF CANNABINOIDS BY REVERSE PHASE CHROMATOGRAPHY



Code: **MET-LAB-001**

Revision No.: **09**

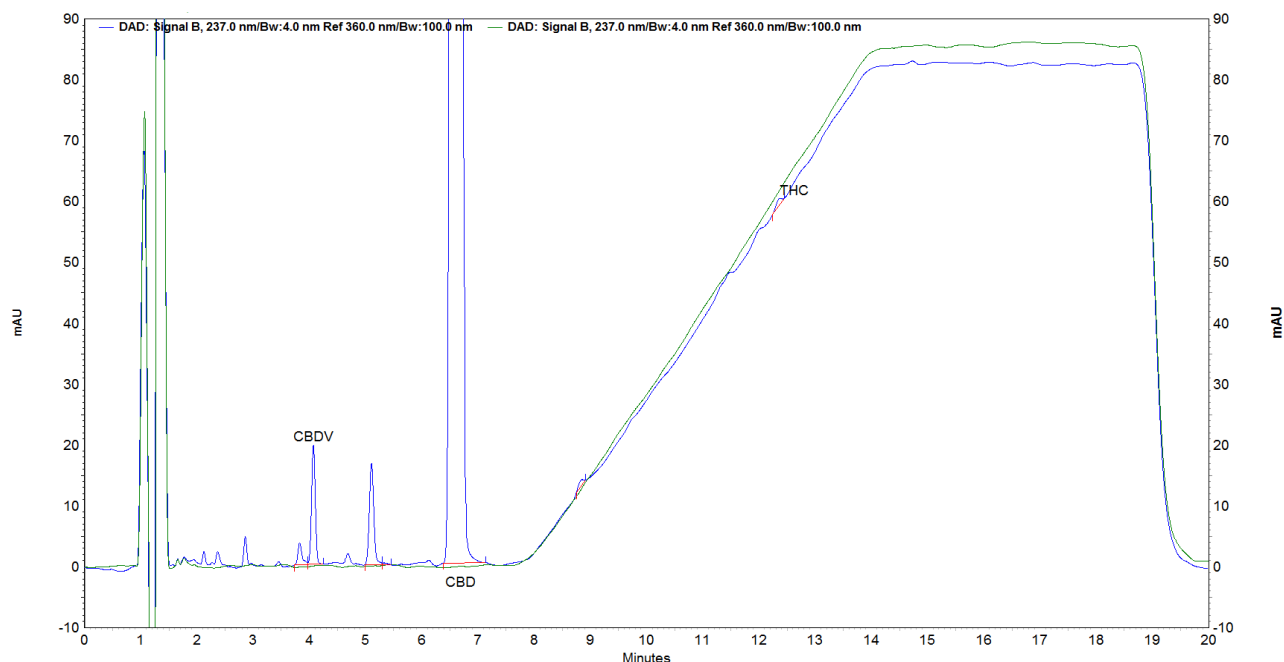


Figure 3: Overlay of the Blank and random sample; signals not present in Blank are integrated, inhibit integration is set for first 3 min, other artifacts present in Blank are also not integrated (procedure MET-LAB-001/01).

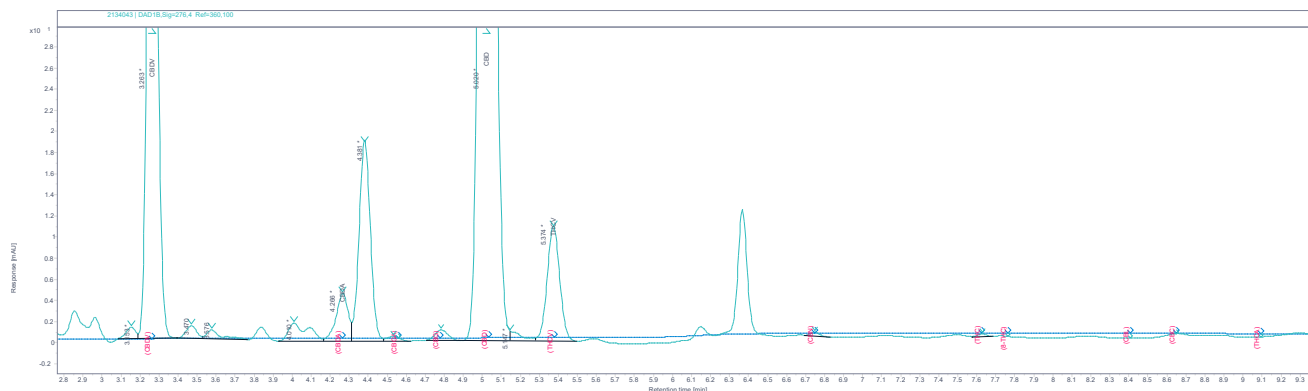


Figure 4: Overlay of the Blank (blue) and random sample (green); signals not present in Blank are integrated, inhibit integration is set for first 3 min, other artifacts present in Blank are also not integrated (procedure MET-LAB-001/02).

Example of CRM injections are shown: Figure 5: procedure MET-LAB-001/01, Figure 6: procedure MET-LAB-001/02 and Figure 7: procedure MET-LAB-001/03.

QUANTITATIVE DETERMINATION OF CANNABINOIDS BY REVERSE PHASE CHROMATOGRAPHY



Code: **MET-LAB-001**

Revision No.: **09**

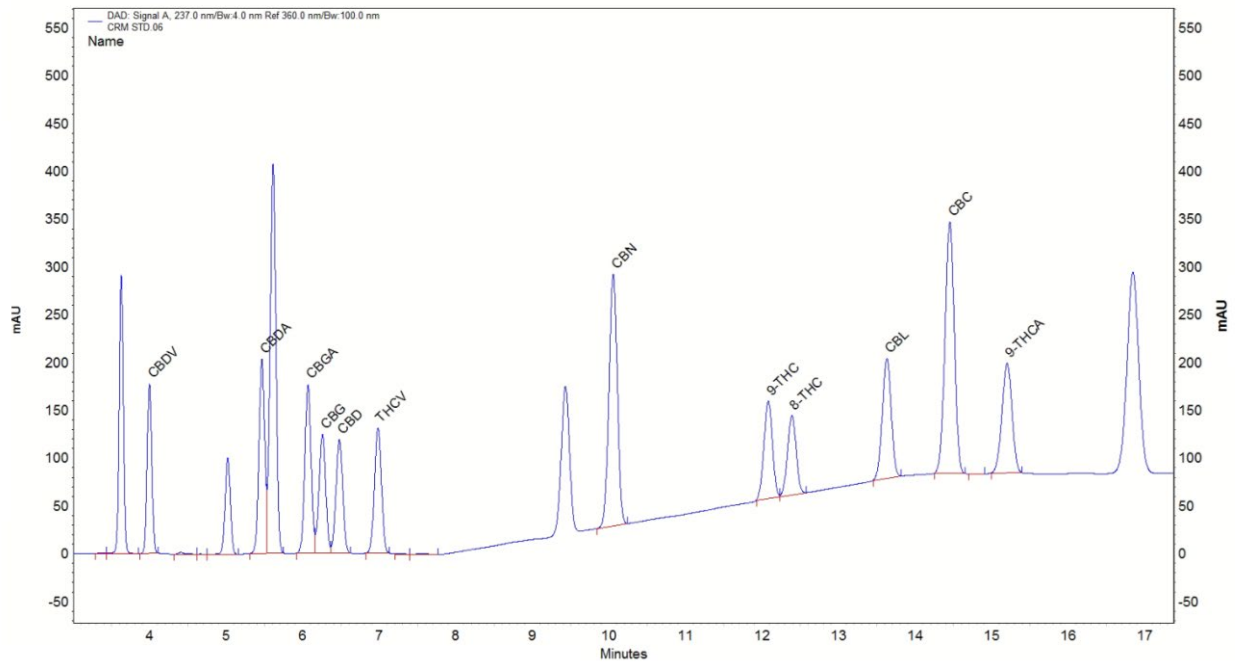


Figure 5: Chromatogram of CRM STD, procedure MET-LAB-001/01.

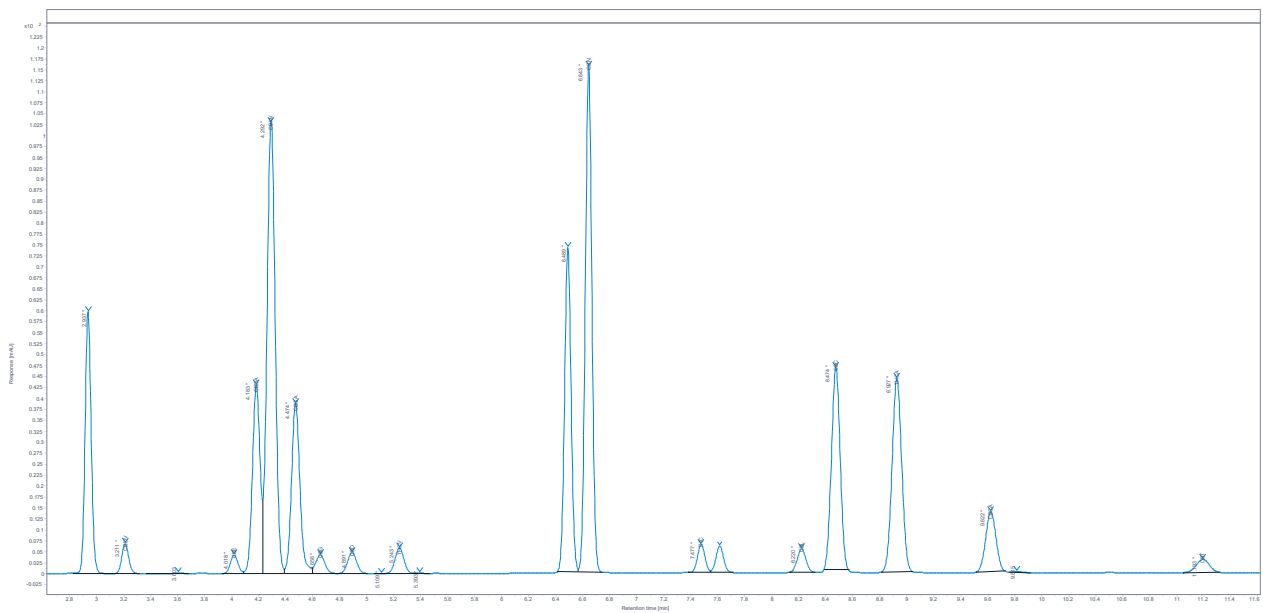


Figure 6: Chromatogram of CRM STD, procedure MET-LAB-001/02.

QUANTITATIVE DETERMINATION OF CANNABINOIDS BY REVERSE PHASE CHROMATOGRAPHY

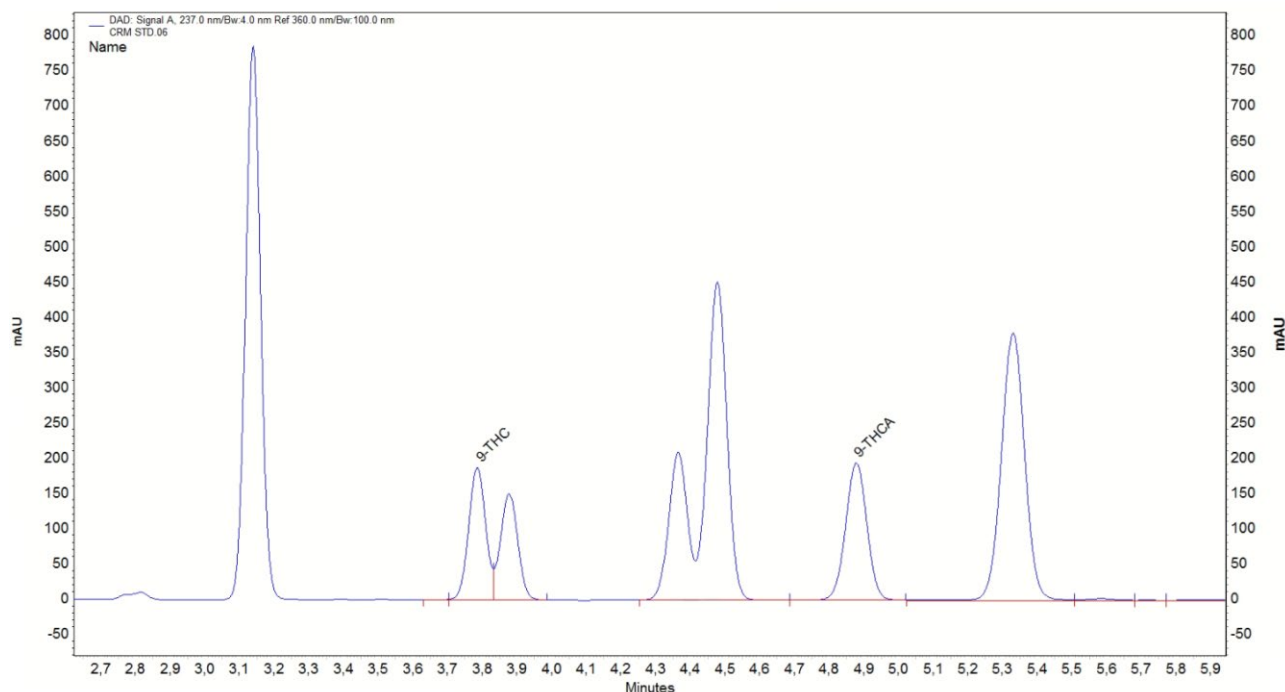


Figure 7: Chromatogram of CRM STD, procedure MET-LAB-001/03.

Baseline:

Generally, draw one baseline from the first to the last peak eluting within the group of the cannabinoid peaks. Set the baseline considering the course of the blank. Select the consistent baseline setting when comparing the results for a given cannabinoid or sample over time. A separate baseline should be set for the baseline separated peaks (see examples: Figure 8 and 9).

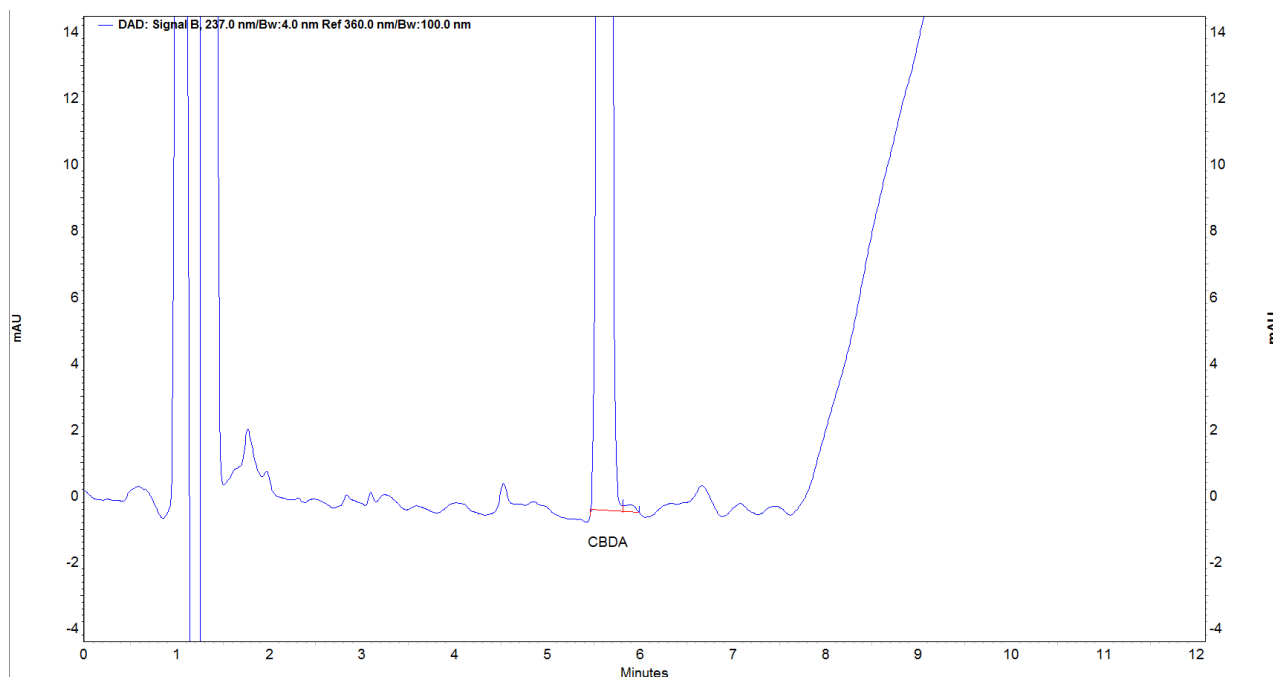


Figure 8: Example for correct baseline determination – baseline separated peak (procedure MET-LAB-001/01).

QUANTITATIVE DETERMINATION OF CANNABINOIDS BY REVERSE PHASE CHROMATOGRAPHY



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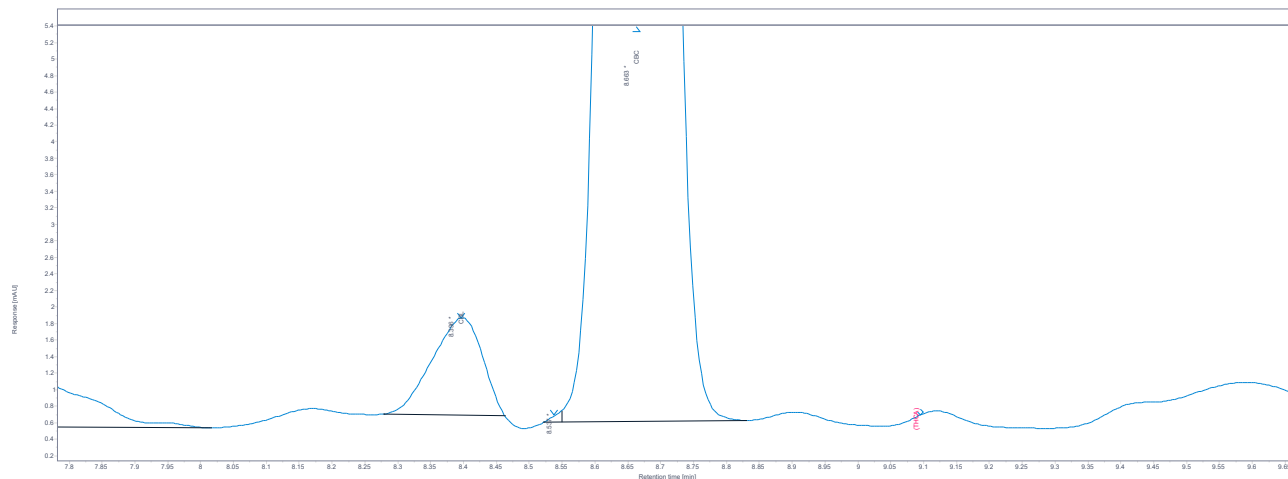


Figure 9: Example for correct baseline determination - baseline separated peak (procedure MET-LAB-001/02).

Within a group of peaks, separate all peaks normally by an orthogonal split. The split should be set if the two peaks are separated by a well-defined valley or at least the maxima of the two peaks are clearly distinguishable. If shoulders of the peak are present, these should also be integrated using orthogonal split at the inflection point (see examples: Figure 10, 11 and 12).

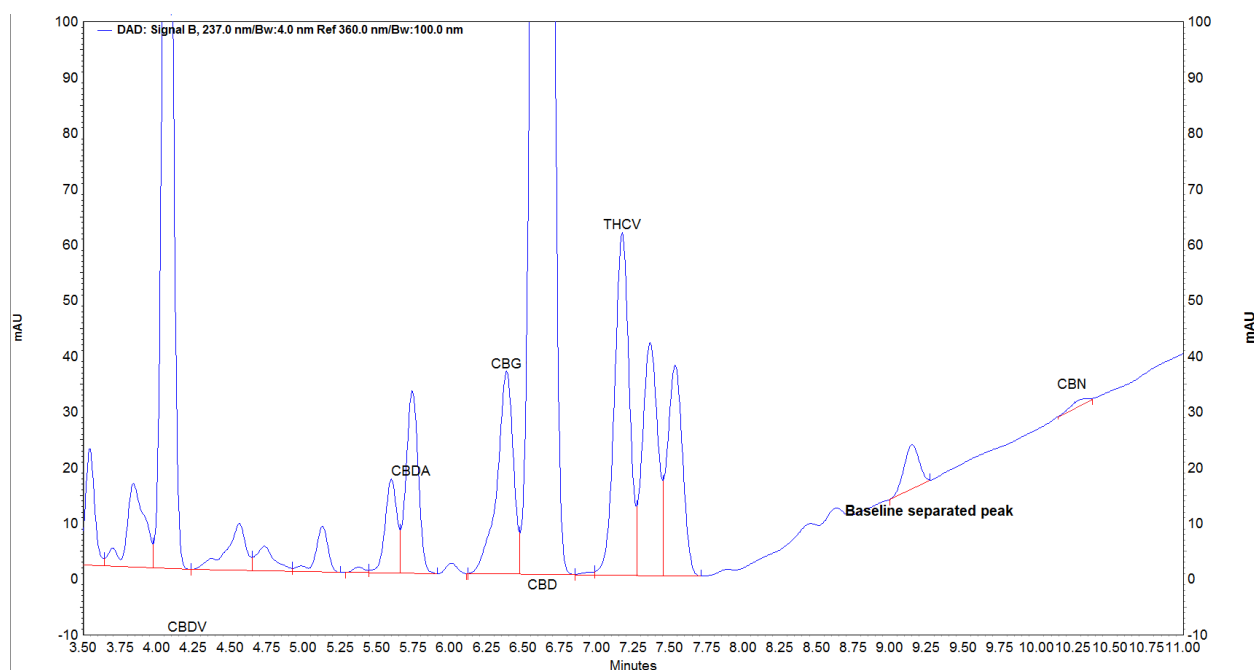


Figure 10: Example when peak is baseline separated and when peaks in the group are separated with a valley (procedure MET-LAB-001/01).

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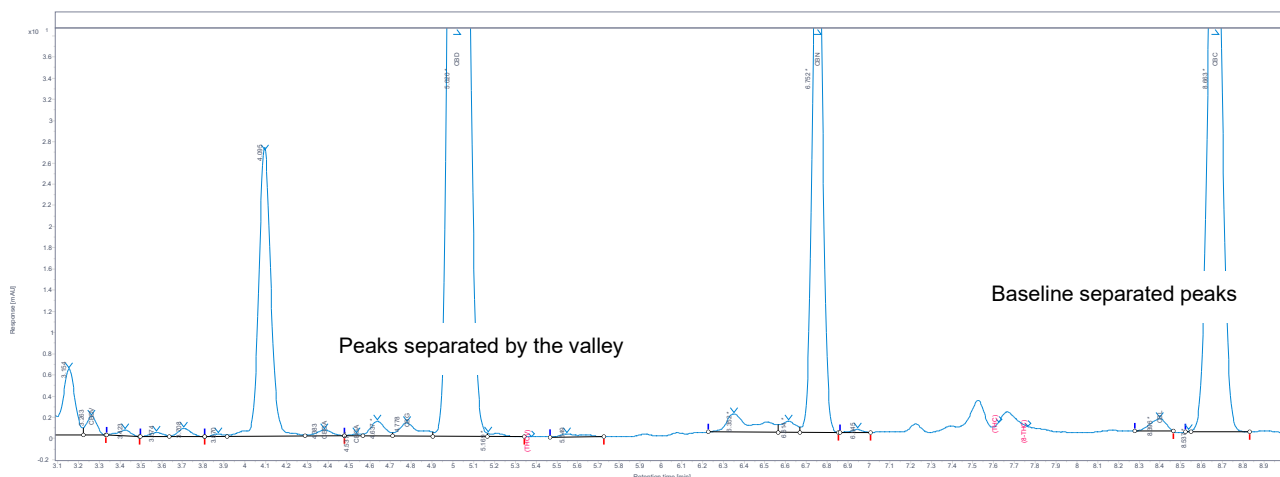


Figure 11: Example when peak is baseline separated and when peaks in the group are separated with a valley (procedure MET-LAB-001/02).

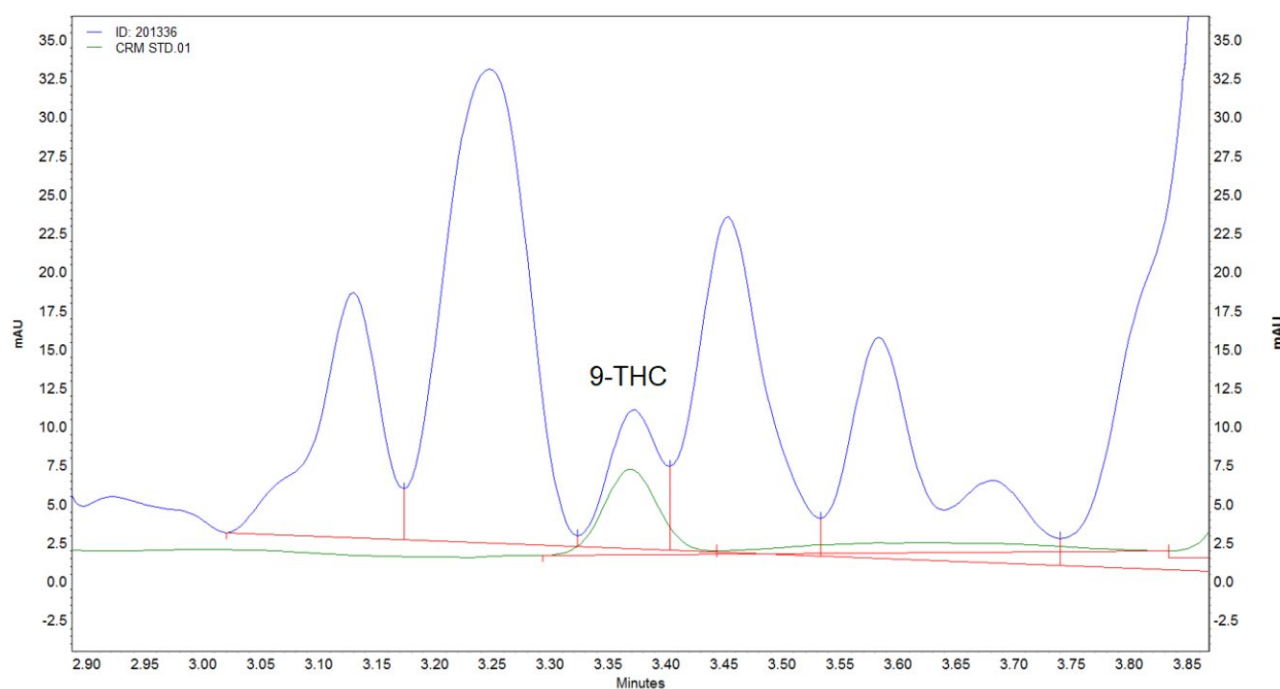


Figure 12: Example for correct integration, when peaks are separated with the valley (procedure MET-LAB-001/03).

If the integration of the whole range is not possible due to the baseline fluctuation or carry over from previous injection and the sample baseline is not adequate (as seen on the Figure 13), the injection of the sample is not valid and sample needs to be re-injected. If the stability of the sample is questionable, sample needs to be re-prepared and re-analyzed (prepared sample is stable up to 1 week at 2-8°C).

QUANTITATIVE DETERMINATION OF CANNABINOIDS BY REVERSE PHASE CHROMATOGRAPHY

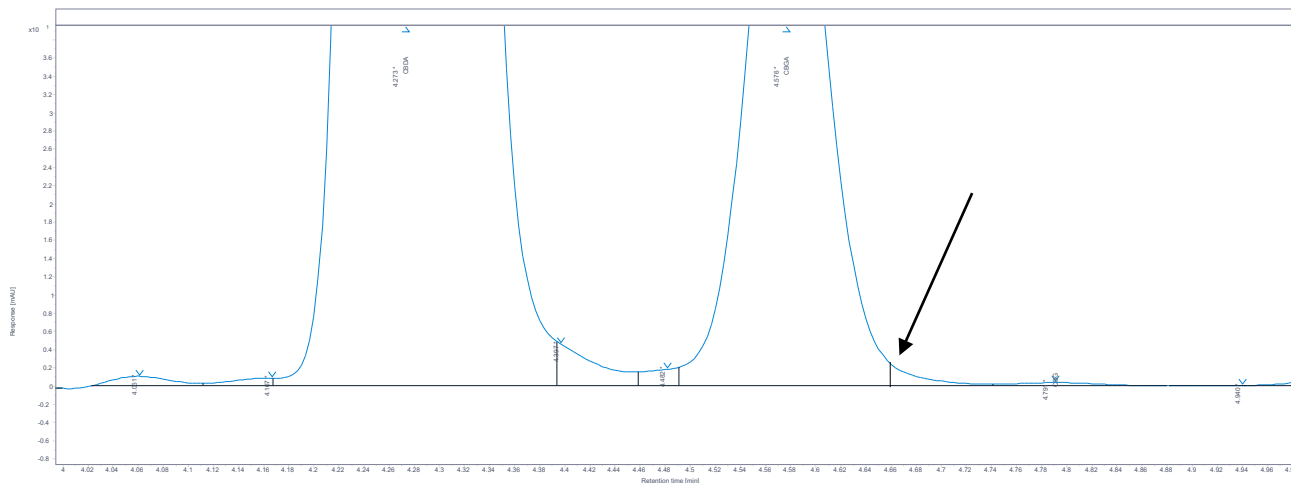


Figure 15: Example when no valley is present but CBDA and unknown shoulder can be split - shoulder is distinguishable (procedure MET-LAB-001/02).

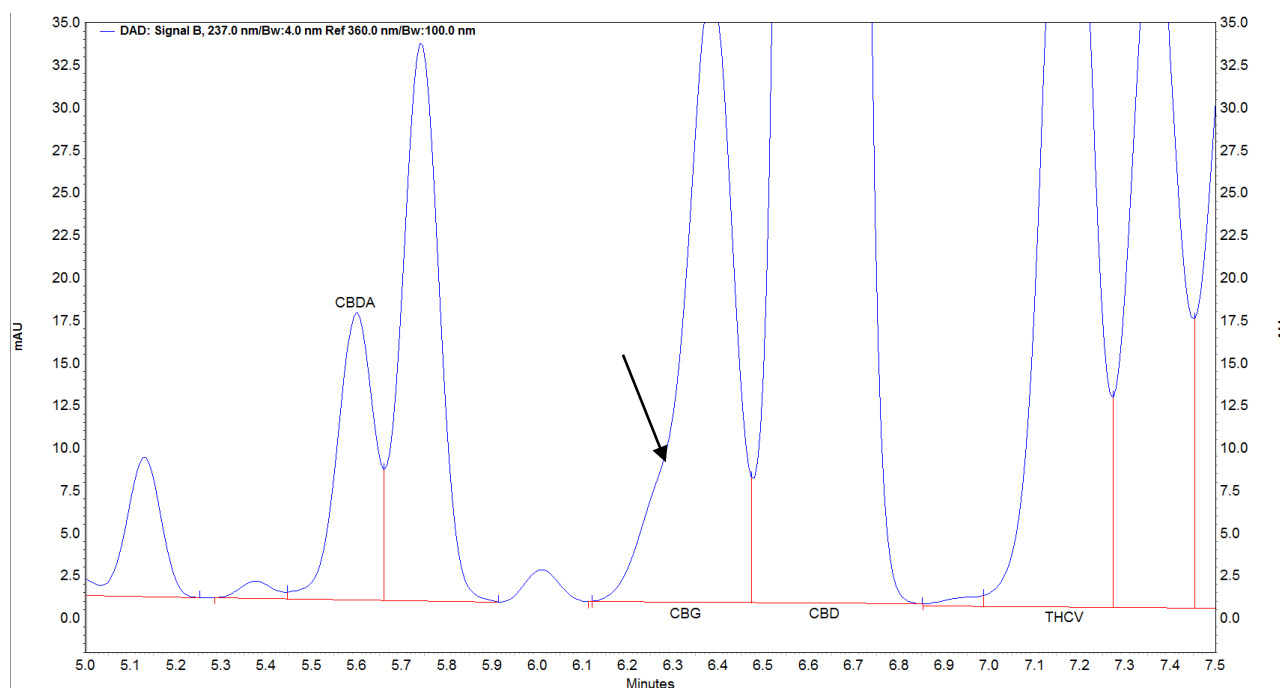


Figure 16: Example when no valley or inflection point is distinguishable - shoulder should not be split (procedure MET-LAB-001/01).

Peak labeling:

Peaks are identified according to their retention time. If the unknown peak is eluted close to the retention time or in the same retention window of the known cannabinoid, the overlay comparison of the chromatogram with the QCS sample must be done (see example: Figure 17 and 18). In extreme cases when even after overlay comparison the identification cannot be determined or the unknown component completely covering the known component the results for that cannabinoid is not valid - in the report the "NA" value is inserted (see example: Figure 19).

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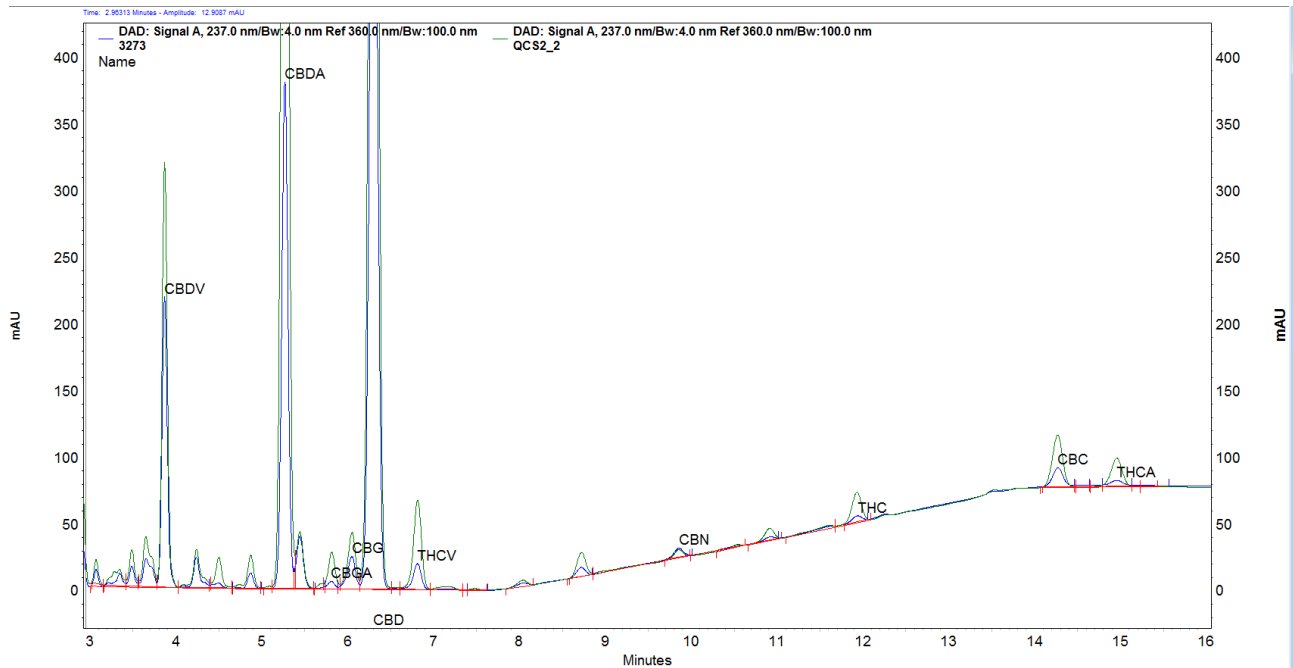


Figure 17: Overlays of the QCS and random sample (procedure MET-LAB-001/01).

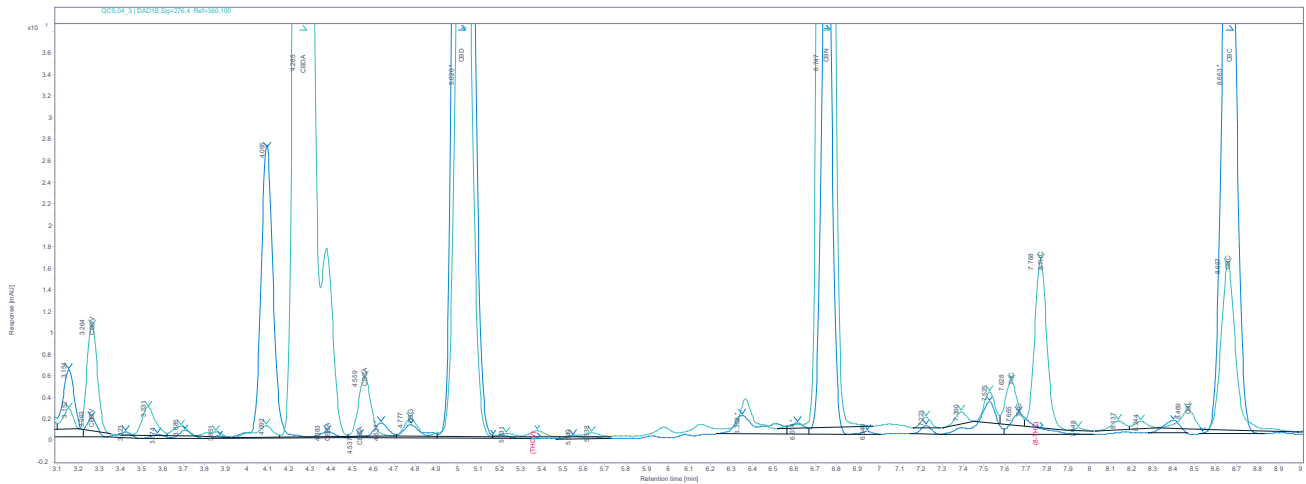


Figure 18: Overlays of the QCS and random sample (procedure MET-LAB-001/02).

QUANTITATIVE DETERMINATION OF CANNABINOIDS BY REVERSE PHASE CHROMATOGRAPHY

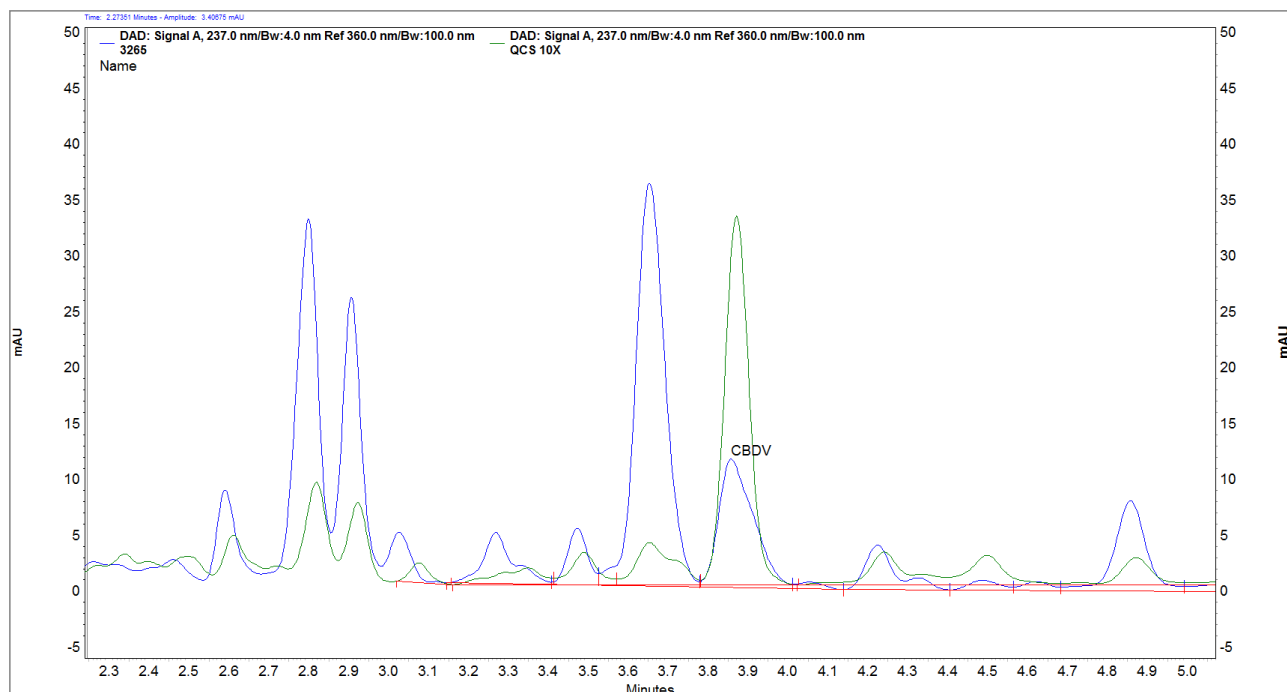


Figure 19: Overlay of the CBDV component in the QCS (green) and sample (blue); CBDV peak in the sample is unsymmetrical, thus the CBDV cannot be determined (value NA in the report is inserted).

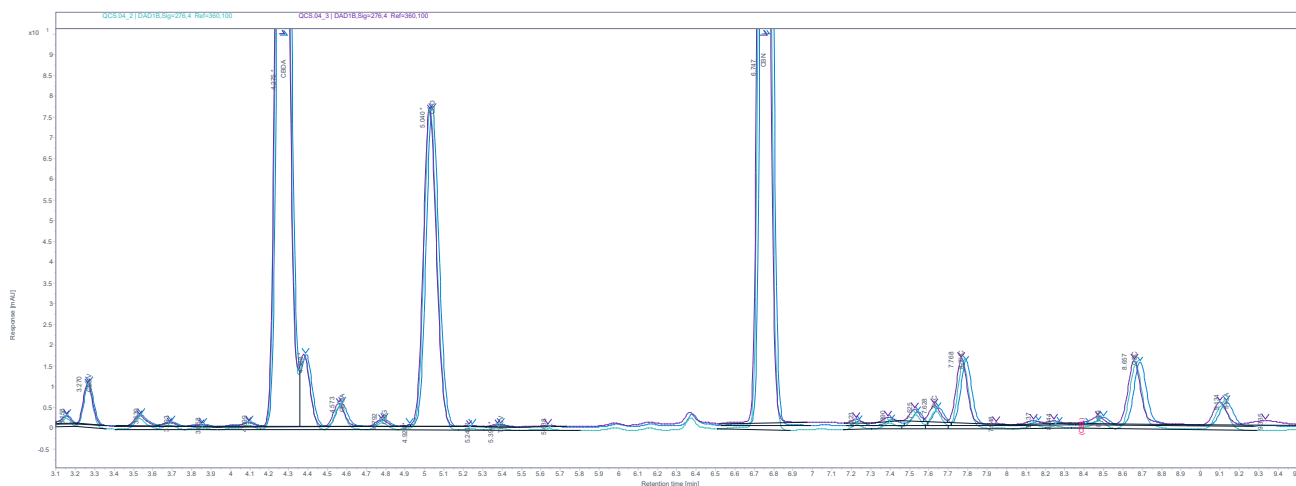


Figure 20: Overlays of the 3 injections of QCS in the same sequence (procedure MET-LAB-001/02).

11. REFERENCES

- DR-001 - Ocena stabilnosti LOQ raztopine
- DR-004 - Referenčni materiali za spremljanje veljavnosti analiz
- DR-005 - Ocena stabilnosti MFB in vpliv povečane vsebnosti vode
- DR-008 - Vpliv koncentriranja vzorcev na določitev vsebnosti kanabinoidov
- DR-011 - Meja detekcije za metodo MET-001
- NZD-LAB-008 - Navodilo za delo s programsko opremo OpenLAB CDS
- SOP-LAB-001 - Zagotavljanje veljavnosti rezultatov
- SOP-LAB-009 - Delovne naloge, odgovornosti in pooblastila
- SPC-LAB-001 - Analitski laboratoriji
- RP-VP-004 - Analytical method validation report for method MET-001

QUANTITATIVE DETERMINATION OF CANNABINOIDS BY REVERSE PHASE CHROMATOGRAPHY

- RP-VP-007 - Analytical method validation report for method MET-002-01
- RP-VP-011 - Analytical method validation report for method MET-003-01
- RP-VP-LAB-22-002 - Analytical method validation report for quantitative determination of CBNV, CBCA and CBT
- RP-VP-LAB-22-003 - Analytical method validation report for conformation of Δ -9-THC and Δ -9-THCA
- VP-01 - Analytical method validation protocol for quantitative determination of cannabinoids by reverse phase chromatography
- VP-005 - Analytical method validation protocol for method MET-002 -01
- VP-011 - Analytical method validation protocol for method MET-003 -01
- VP-013 - Analytical method validation protocol for conformation of Δ -9-THC and Δ -9-THCA
- ISO 9001:2015 - Sistemi vodenja kakovosti - Zahteve
- ISO/IEC 17025:2017 - Splošne zahteve za usposobljenost preskuševalnih in kalibracijskih laboratorijev
- ISO 22000:2018 - Sistemi vodenja varnosti živil - Zahteve za vsako organizacijo v prehranski verigi
- ISO 22716:2007 - Kozmetika - Dobra proizvodna praksa (GMP) - Smernice za dobro proizvodno prakso
- Smernice z dne 19. marca 2015 o načelih dobre distribucijske prakse za zdravilne učinkovine za zdravila za uporabo v humani medicini (2015/C 95/01) (GDP)
- Commission regulation (EU) 2022/1393 from 11th of August 2022; amending Regulation (EC) No 1881/2006 as regards maximum levels of delta-9-tetrahydrocannabinol (Δ -9-THC) in hemp seeds and products derived therefrom

12. APPENDIX

- Appendix 1: Logbook

13. HISTORY OF THE DOCUMENT

Revision Number	Amendments	Issue date	Changed with new version (date, signature)
01	First issue	23. 05. 2019	30. 08. 2019
02	Statement that the method is validated with the reference on the validation report was added. The stabilities of solutions were updated, according to the validation report. Correction of the concentration range of the method as a result a different preparation of the dilutions - the dilution steps for the samples were updated according to the validation report. More detail instructions were added for the sample preparation. Accuracy of the method must be evaluated every 6 months, using the certified reference standards. Method re-calibration is required each year, after the re-qualification of the HPLC instrument. Chapter 8.3 Result reporting was added - detail instruction which dilution is taken into account and how are results reported. Minor typos throughout of the document were corrected.	30. 08. 2019	30. 06. 2020
03	In Table 2 and Table 3 isopropanol was added, which is used for HPLC system.	30. 06. 2020	09.10.2020

**QUANTITATIVE DETERMINATION OF CANNABINOIDS BY
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Revision Number	Amendments	Issue date	Changed with new version (date, signature)
	In Table 5 the vials and vial caps from KEFO can also be used as proven during validation study. The reagents and solution stability were updated in chapter 6.1. The chapter 6.3 was updated in accordance to the SOP-LAB-001. In chapter 6.4 the statement that the sample preparation must be repeated in cases of electrical outages - if the prepared samples are not stored at adequate temperatures. The quality control samples were update, now in every analysis also the CRM standards are used for quality control. The sequence set-up was updated - now all quality control samples are included. The chromatogram of CRM STD was added. The Appendix 2 was added with the Logbook details for sample preparation. Minor typos throughout of the document were corrected.		
04	The title was corrected due to the typographical error. Instruction for concentrating (when more than 100 mg od sample is weighted) were added to the chapter 6.4 (sample preparation protocol). The instructions for reporting when concentrating is applied were added. The LOQ values for the method was changed from 0.03 % to 0.3 µg/ml.	09. 10. 2020	03. 12. 2020
05	The 8-THC and CBL were added for quantification of cannabinoids, therefore the chemicals and the procedure for preparation of calibration standards were updated accordantly. The instructions for future validation of the additional cannabinoids were written. The stability of the quality control samples was evaluated through the control charts and the stability of prepared solutions were updated. The instructions for re-injection of the sample when the baseline fluctuation or carry over is present were added	04. 12. 2020	07. 12. 2021
06	Code of the document changed from MET-001 to MET-LAB-001. More detailed specifications were added for chemicals and reagents, CRMs, consumables and equipment/software; additional can be used in accordance to SPC-LAB-001. When different chemicals, reagents or CRMs is used their suitability must be confirmed by validation. Resolution tracking in REF STD was added, appendix 2 was corrected accordantly. Sample preparation instruction was updated to include the 10x and 100x concentrated sample preparation - 100x concentrated sample can be prepared only for certain matrices. The additional sample preparation protocols were updated for balms and gummies as confirmed during validation.	07. 12. 2021	24. 10. 2022
07	Analytical methods MET-LAB-001, MET-LAB-002 and MET-LAB-003 were merged into a single analytical method MET-LAB-001, where previous methods are described as individual procedures of the method MET-LAB-001. The chapters of the document were arranged regarding to the instructions. The general instructions which are the same for all the procedures are at the beginning of the document and	24. 10. 2022	22.12.2022

**QUANTITATIVE DETERMINATION OF CANNABINOIDS BY
REVERSE PHASE CHROMATOGRAPHY**



Code: **MET-LAB-001**

Revision No.: **09**

Revision Number	Amendments	Issue date	Changed with new version (date, signature)
	individual instructions for each procedure are written under each procedure. More detailed chromatograms were added at the end of the document to better understand the correct integration of the samples.		
08	The abbreviation for Cannabivarin was updated from CBNV to CBV through the document. The equation for calculation of Total THC from Δ -9-THCA and Δ -9-THC was added in Chapter 1, following the EU Regulation No 2022/1393.	22.12.2022	
09	Reference for new validation report for cannabinoid CBE was added and Item number for CBE (from reference standard to certified reference material) was replaced.		

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Code: **OBR1-MET-LAB-001**

Revision No.: **09**

APPENDIX 1: Logbook

ANALYSIS ID: PHL_RPC_16C_	PREPARATION DATE:
PROCEDURE: MET-LAB-001/	SIGNATURE:

Page: ___ of ___

1. MOBILE PHASES

2. SAMPLE DILUTION BUFFER /BLANK

MPA: PR-0_, p.:_, date:_____ **Blank 1:** PR-0_, p.:_, date:_____, ID:_____ vial:_____

MPB: PR-0_, p.:_, date:_____ ID:_____ **Blank 2:** PR-0_, p.:_, date:_____, ID:_____ vial:_____ (optional)

3. SAMPLE PREPARATION:

Follow the instruction to enter the data in OpenLAB: **NZD-LAB-008** in **NZD-LAB-010**.

#	Sample ID	Weight [mg]	Dilutor 1	Multiplier 1	Dilutor 2	Multiplier 2	Analyst (Signature and date)	Data entry in OpenLAB (Date and signature)	Data review in OpenLAB (Date and signature)
1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
13									
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**QUANTITATIVE DETERMINATION OF CANNABINOIDS BY
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APPENDIX 1: Logbook

Code: **OBR1-MET-LAB-001**

Revision No.: **09**

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Additional remarks regarding the sample preparation:

4. CONTROL SAMPLES

Information regarding the control samples are in the corresponding control charts.

Control sample	[☐ / ☐]	Aliquot put out of the freezer [2-8°C]	Aliquot expiration date
LOQ	☐		
REF STD 4xR	☐		
REF STD 40xR	☐		
CRM STD	☐		
QCS	☐		
QCS 10xR	☐		
CRM STD_9THC_9THCA	☐		

5. PROCESSING METHOD ON HPLC SYSTEM: _____

6. ANALYSIS VALIDITY:

Validity of analysis is evaluated through the control charts, following the criteria in **SOP-LAB-001**.

6.1 Sample dilution buffer: Blank is without peaks in the RT window of cannabinoids: YES / NO

Comment:

6.2 HPLC system sensitivity: S/N ≥ 10? YES / NO Value: _____

Comment:

6.3 Resolution between CBD and CBG: _____; Validity: YES / NO

Comment:

6.4 Validity of the QCS sample in the beginning, every 15 injections and in the end of the sequence? YES / NO

Comment:

6.5 Validity of the CRM STD and/or CRM STD_9THC_9THCA? YES / NO

Comment:

6.6 Validity of the REF STD? YES / NO

Comment:

THE CRITERIA FOR VALIDITY OF ANALYSIS ARE MET, ANALYSIS IS VALID: YES / NO

7. ANALYSIS REVIEW AND APPROVAL (Date and signature): _____

Comment: