# Metabolite analysis of Cannabis sativa L. by NMR spectroscopy

Flores-Sanchez, I.J.; Choi, Y.H.; Verpoorte, R.; Kaufmann, M.; Klinger, C.

# Chapter 27

# Metabolite Analysis of *Cannabis sativa* L. by NMR Spectroscopy

**Isvett Josefina Flores-Sanchez, Young Hae Choi, and Robert Verpoorte**

## Abstract

NMR-based metabolomics is an analytical platform, which has been used to classify and analyze *Cannabis sativa* L. cell suspension cultures and plants. Diverse groups of primary and secondary metabolites were identified by comparing NMR data with reference compounds and/or by structure elucidation using $^{1}$H-NMR, *J*-resolved, $^{1}$H-$^{1}$H COSY, and $^{1}$H-$^{13}$C HMBC spectroscopy. The direct extraction and the extraction by indirect fractionation are two suitable methods for the *C. sativa* sample preparation. Quantitative analyses could be performed without requiring fractionation or isolation procedures.

**Key words:** *Cannabis sativa*, Metabolomics, Multivariate data analysis, Nuclear magnetic resonance, Principal component analysis

## 1. Introduction

*Cannabis sativa* L. is an annual dioecious plant which produces several metabolite groups from plant primary and secondary metabolism. Amino acids, fatty acids, sugars, and steroids are some examples from primary metabolites. More than 247 compounds have been identified as secondary metabolites (1–3). They have been grouped into six groups of secondary metabolism: cannabinoids, flavonoids, stilbenoids, terpenoids, lignans, and alkaloids. Several analytical platforms have been used to analyze, identify, and quantify the different metabolites present in this plant (4–12). From traditional techniques, either spectroscopic or chromatographic as UV, TLC, CPC, HPLC, GC, MS, IR, NMR, to high-throughput techniques as hyphenated methods (LC–MS, GC–MS, Py-GC–MS, GLC). Although NMR is a common tool used for structural elucidation

Michael Kaufmann and Claudia Klinger (eds.), *Functional Genomics: Methods and Protocols*,
Methods in Molecular Biology, vol. 815, DOI 10.1007/978-1-61779-424-7_27, 

of compounds new applications are being explored. $^{1}$H-NMR spectroscopy is an analytical platform in the field of plant metabolomics (13, 14). It is known that metabolomics has facilitated an improved understanding of cellular responses to environmental changes (15–17). For NMR-based metabolomics, the analysis allows the simultaneous detection of diverse groups of primary and secondary metabolites. The signals in an NMR spectrum are proportional to their molar concentrations, so a direct comparison of concentrations of all compounds is possible. Using two-dimensional NMR measurements, many signals can be identified (18–20). NMR-based metabolomics from Cannabis plants and cell suspension cultures have been reported for classification of *C. sativa* cultivars and cell lines, and for the analysis of its metabolism under stress conditions (21–23).

## 2. Materials

### 2.1. Cannabis Plants and Cell Suspension Cultures

1. Air-dried or fresh flowers and leaves of *C. sativa* L. (Stichting Institute for Medicinal Marijuana, Rotterdam, The Netherlands).
2. Seeds of *C. sativa* (The Sensi Seed Bank, Amsterdam, The Netherlands and Dr. D. Watson, HortaPharm, Amsterdam, The Netherlands) for establishing cell culture lines.
3. Cannabis cell suspension cultures.
4. Erlenmeyer (EM) flasks (250 ml) containing 50 ml of Murashige and Skoog (MS) basal medium supplied with 10 mg/L thiamine hydrochloride, 1 mg/L pyridoxine hydrochloride, 1 mg/L nicotinic acid, 1 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid), 1 mg/L kinetin and 30 g/L sucrose.
5. MS basal medium: $NH_4NO_3$ (1,650 mg/L), $KNO_3$ (1,900 mg/L), $CaCl_2{\cdot}2H_2O$ (440 mg/L), $MgSO_4{\cdot}7H_2O$ (370 mg/L), $KH_2PO_4$ (170 mg/L), KI (0.83 mg/L), $H_3BO_3$ (6.2 mg/L), $MnSO_4{\cdot}4H_2O$ (22.3 mg/L), $ZnSO_4{\cdot}7H_2O$ (8.6 mg/L), $Na_2MoO_4{\cdot}2H_2O$ (0.25 mg/L), $CuSO_4{\cdot}5H_2O$ (0.025 mg/L), $CoCl_2{\cdot}6H_2O$ (0.025 mg/L), $FeSO_4{\cdot}7H_2O$ (27.8 mg/L), $Na_2EDTA{\cdot}2H_2O$ (37.3 mg/L), myo-inositol (100 mg/L) and glycine (2 mg/L).

### 2.2. Elicitors

1. 10 mg/ml yeast extract (Bacto™ Brunschwig Chemie, Amsterdam, The Netherlands).
2. 8 mg/ml *Pythium aphanidermatum* (Edson) Fitzp. (313.33, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands).
3. 8 mg/ml *Botrytis cinerea* Pers. (Stichting Institute for Medicinal Marijuana, Rotterdam, The Netherlands).

4. 1 mM salicylic acid.
5. 0.3 mM methyl-jasmonic acid.
6. 100 µM jasmonic acid.
7. 0.1 mg/ml *Citrus fruits* pectin (87% galacturonic acid and 8.7% methoxy groups, Sigma).
8. 84 µg/ml Cannabis pectin extract.
9. 150 µg/ml sodium alginate (Fluka, Buchs, Switzerland).
10. 100 µM $AgNO_3$.
11. 100 µM $CoCl_2$.
12. 100 µM $NiSO_4$.
13. Exposition to UV-irradiation using UV 302 and 366 nm lamps (Vilber Lourmat, France) for 30 s or 30 min.

### 2.3. Indirect Fractionation Method

1. MeOH:Water (1:1, *v/v*).
2. $CHCl_3$.
3. Ethyl acetate.

### 2.4. Direct Extraction Method

1. MeOD: 90 mM $KH_2PO_4$ buffer in $D_2O$ (pH 6.0; adjusted with 1 M NaOD) (1:1, *v/v*) containing 0.1% TMSP-$d_4$ [3-(trimethylsilyl) propionic-2,2,3,3-$d_4$ acid sodium salt, 99 atm.% D] (*w/w*).
2. $CDCl_3$ containing 0.01% HMDSO-$d_{18}$ (hexamethyl-$d_{18}$-disiloxane, 99 atm.% D) (*w/w*).

### 2.5. Additional Material

1. Liquid nitrogen.
2. Eppendorf tubes, 2 ml.
3. Cap test tubes, 15 ml (plastic and glass).
4. Evaporating flask, 25 ml.
5. Pasteur pipettes.
6. NMR tubes, 5 mm.

### 2.6. Equipment

1. Freeze-dryer for sample drying.
2. Ultrasonicator.
3. Refrigerated centrifuge.
4. Rotary evaporator.
5. 500 MHz Bruker NMR spectrometer (DMX500, Bruker) equipped with a 5 mm TXI probe and a *z*-gradient system or similar instrument.

### 2.7. Software

1. Microsoft Excel™.
2. AMIX version 3.7 (Bruker Biospin) for bucketing NMR data.
3. SIMCA-P version 11.0 (Umetrics AB, Umea, Sweden) or comparable software for multivariate analysis.

## 3. Methods

### 3.1. Elicitation of Cannabis Cell Suspension Cultures

1. Inoculate 5 g of fresh Cannabis cells into an EM flask containing fresh MS medium.
2. Grow the cultures at 110 rpm and 25°C under a light intensity of 1,000–1,700 lx.
3. Five days after inoculation, add elicitors to Cannabis cell cultures or expose them to UV-irradiation (see Notes 1–3).
4. Collect the Cannabis cells using a Büchner funnel at different time periods (see Note 4).
5. Freeze the cells using liquid nitrogen and store them at −80°C (see Note 5).
6. Keep on freezing the medium of cell cultures if you plan to analyze it too.

### 3.2. Lyophilization of Plant Material

1. Grind the frozen plant material (Cannabis cells or tissues) using a precooled pestle and mortar under liquid nitrogen (see Note 6).
2. Transfer the grounded plant material into a plastic tube and keep it in dry ice, liquid nitrogen or freezer before lyophilization.
3. Place the samples in a freeze-dryer for 1–3 days (see Notes 7–8).

### 3.3. Extraction Method by Indirect Fractionation

1. Weigh 1 g (fresh) or 100 mg (dry) Cannabis plant material in a glass cap test tube.
2. Add 4 ml of MeOH:$H_2O$ and 4 ml of $CHCl_3$ (see Note 9).
3. Mix for 30 s using a vortex at room temperature.
4. Ultrasonicate for 10 min at room temperature.
5. Centrifuge at 4°C and 9,000 × *g* for 20 min to obtain a clear supernatant.
6. Separate $CHCl_3$ fraction (lower phase) and MeOH:Water fraction (upper phase) using Pasteur pipettes and transfer to evaporating flasks.
7. Repeat a second extraction (solvent addition, mixing, sonication, centrifugation, and separation; see Note 10).
8. Evaporate each fraction using a rotary evaporator (see Note 11).
9. Resuspend in 1 ml of $CDCl_3$ (with 0.01% HMDSO-$d_{18}$) and 1 ml of MeOD:$KH_2PO_4$ buffer (with 0.1% TMSP-$d_4$), respectively. Mix for 1–2 min on a vortex (see Note 15).
10. Transfer the MeOD:$KH_2PO_4$ buffer solution to an Eppendorf tube and centrifuge for 5 min at maximum speed.
11. Load the MeOD:$KH_2PO_4$ buffer solution to 5-mm NMR tube (see Note 12).

12. Make a filter using a Pasteur pipette and tissue (Kimwipes™ or wool).
13. Load the filter on a 5-mm NMR tube.
14. Filter directly the $CDCl_3$ solution into the NMR tube (see Note 12).

### 3.4. Direct Extraction Method

1. Weigh 50–150 mg (dry or fresh) Cannabis plant material in a 2.0-ml Eppendorf tube or glass cap test tube.
2. Add 1 ml of MeOD:$KH_2PO_4$ buffer (with 0.1% TMSP-$d_4$; see Note 15) and 1 ml of $CDCl_3$ (with 0.01% HMDSO).
3. Mix for 30 s using a vortex at room temperature.
4. Ultrasonicate for 10 min at room temperature.
5. Centrifuge at 4°C and 9,000 × *g* (glass cap test tube) or 13,800 × *g* (Eppendorf tube) for 20 min to obtain a clear supernatants.
6. Separate $CDCl_3$ fraction (lower phase) and MeOD:$KH_2PO_4$ buffer fraction (upper phase) using Pasteur pipettes and transfer to Eppendorf tubes.
7. Centrifuge for 1 min at 16,200 × *g* at room temperature.
8. Transfer 800 µl of the MeOD:$KH_2PO_4$ buffer solution to a clean 5-mm NMR tube and 800 µl of the $CDCl_3$ solution to a clean 5-mm NMR tube (see Note 12).

### 3.5. Extraction Method for Cell Culture Medium

1. Put 10 ml cell culture medium in a separating funnel.
2. Add 10 ml ethyl acetate and shake the separating funnel.
3. Transfer the ethyl acetate phase into a beaker.
4. Repeat a second extraction using 10 ml ethyl acetate.
5. Dry the ethyl acetate phase with $Na_2SO_4$.
6. Transfer the ethyl acetate phase to an evaporating flask.
7. Evaporate using a rotary evaporator (see Note 11).
8. Resuspend in 1 ml of MeOD and mix for 1–2 min on a vortex.
9. Load 800 µl of MeOD solution to a 5-mm NMR tube (see Note 12).

### 3.6. NMR Measurement

1. Load the 5-mm NMR tube with your sample into the spectrometer (see Notes 13–14).
2. Set the sample temperature to 25°C and wait for thermal equilibration.
3. Tune and match the NMR tube.
4. Lock the spectrometer frequency to the deuterium resonance arising from the NMR solvents (MeOD or $CDCl_3$).
5. Shim the sample using either a manual or an automated method.

6. Determine the frequency of the water resonance and set the center of the spectrum to this frequency.
7. *For Standard 1H-NMR spectroscopy*: Set up pulse sequence comprising (relaxation delay-90°-acquire), where pulse power is set to achieve a 90° flip angle, 4.0 kHz spectral width and water pre-sat applied during 1.0-s relaxation delay (see Note 23).

   *Processing parameters*: Zero-fill to 64 k data points, apply exponential line broadening of 0.3 Hz. Free induction decay signals are transformed by Fourier with LB = 1.0 Hz, GB = 0 and PC = 1.0. After Fourier transformation, manually phase spectrum (zero and first phase), correct baseline, and calibrate the spectrum by setting TMPS peak at 0.00 ppm (for methanol:water fraction), MeOD peak at 3.30 ppm (for methanol fraction), $CDCl_3$ peak at 7.26 ppm or HMDSO peak at 0.07 ppm (for chloroform fractions). Record 128 scans for each sample (see Notes 16–21, 24 and 25).
8. *For J-resolved spectroscopy (homonuclear two-dimensional J-resolved NMR spectroscopy)*: Set up *J*-resolved pulse sequence, two-pulse echo sequence (relaxation delay-90°-[t1/2]–180°-[t1/2]-acquire) with water pre-sat during a relaxation delay of 1.5 s. Acquire FID using data matrix of 64 × 4,096 points covering 66 × 6,361 Hz, with 16 scans for each increment. Zero-fill the data to 128 × 4,096 and apply a sine bell-shaped window function in both dimensions before magnitude mode two-dimensional Fourier transformation. Tilt the resulting spectra along the rows by 45° relative to the frequency axis and symmetrize about the central line along F2. Manually correct baseline and calibrate to the internal standard (see Notes 19–21).
9. *For $^{1}H$-$^{1}H$ COSY (two-dimensional homonuclear $^{1}H$-$^{1}H$ correlated NMR spectroscopy)*: Use a phase sensitive/magnitude mode standard three pulse sequence with pre-saturation during relaxation delay of 1.0 s. A data matrix of 512 × 4,096 points covering 6,361 × 6,361 Hz, record with eight scans for each increment. Zero fill data to 4,096 × 4,096 points and apply a sine 2 bell-shaped window function shifted by /2 in the F1 and /4 in the F2 dimension before States-TPPI type two-dimensional Fourier transformation. Manually phase all spectra, correct baseline, and calibrate to the internal standard).
10. *For $^{1}H$-$^{13}C$ HMBC (two-dimensional heteronuclear multiple bond correlation NMR spectroscopy)*: Use a data matrix of 254 × 4,096 points covering 27,164 × 6,361 Hz with 256 scans for each increment with a relaxation delay of 1.0 s. The data should be linear to 512 × 4,096 points using 32 coefficients before magnitude type two-dimensional Fourier transformation and apply a sine bell-shaped window function shifted by /2 in

the F1 dimension and /6 in the F2 dimension. Calibrate all spectra according to the internal standard ($^{1}$H: TMSP = 0 ppm and $^{13}$C: $CD_3OD$ = 49.0 ppm).

### 3.7. Data Processing

1. Convert NMR spectra to an ASCII file using AMIX software. Scale the spectral intensities to HMDSO for the $CHCl_3$ fractions and TMSP for MeOD:Water fractions. For MeOD fractions scale to total intensity.
2. Integrate the peaks into a small bin (bucket) from 0.04 ppm to avoid signal fluctuation by pH or concentration changes.
3. Delete solvent signals (δ3.28–3.34 for MeOD, δ4.6–5.8 for water, and δ7.18–7.30 for $CDCl_3$).
4. Copy the ASCII data to an Excel table to identify and classify your samples according to your requirements.
5. Perform the principal component analysis (PCA) or partial least square discriminant analysis (PLS-DA) using the SIMCA-P software according to the user guides. Select the Pareto scaling for a variance numerically equal to its initial standard deviation.
6. Display the score and loading plots.
7. Look for patterns or clusters in the dataset. Identify the metabolites responsible for those differences or similarities among the datasets, either by comparison with NMR signals to reference compounds or by two-dimensional NMR spectra.

### 3.8. Quantitative Analysis

1. Identify the proton signals of the target compounds and the internal standard.
2. Determine the integral of the target and standard peaks (see Note 22).
3. Based on the quantity of the internal standard, the concentration (in μmol/100 mg of dry cell material) is calculated using the following equation (see Note 23):

   Concentration (μmol/100 mg dry weight) = [integral of target compound/integral of internal standard] × [number of protons from internal standard/number of protons from target compound] × quantity of internal standard (μmol).
4. Based on the weight of the internal standard, the concentration (in μg/100 mg of cell material) is calculated using the following equation (see Note 23):

   Concentration (μg/100 mg dry weight) = [integral of target compound/integral of internal standard] × [number of protons from internal standard/number of protons from target compound] × [MW of target compound/MW of internal standard] × weight of internal standard (μg).

## 4. Notes

1. Elicitation should be done during the exponential phase of growth.
2. Elicitors should be sterilized by autoclaving or filtration (0.22-μm filter) before adding to cell suspensions.
3. Methyl-jasmonic acid and jasmonic acid can be dissolved in EtOH or in a 30% EtOH solution (*v*/*v*).
4. Use identical harvesting times because the metabolite levels from plants vary throughout the day.
5. Liquid nitrogen should be handled carefully. Always use glasses and gloves.
6. Grinding to fine powder plant material has the advantage of improving the efficiency of extraction.
7. In the freeze-dryer, place uncovered tubes with sample or cover with perforated paper.
8. After lyophilization keep your samples in a dry environment because it can absorb moisture.
9. Prepare a new MeOH:Water (1:1) solution every time that you perform extractions. Do not store it because the ratio between MeOH and water may vary over time.
10. After extraction, a fractionation step by solid phase extraction (SPE) may facilitate identification of secondary metabolite signals on removing primary metabolite signals. Use $C_{18}$ or silica gel cartridges (1 or 3 ml).
11. Extracts can be stored at 4°C.
12. Clean NMR tubes using in the following order of solvents: water, ethanol, methanol, diclorometh ane, and acetone. Use an NMR tube cleaner.
13. Do not expose NMR tubes to high temperatures during drying because they may lose their properties of uniformity and/or concentricity.
14. On cleaning NMR tubes, be careful not to scratch and not to use reagents that can attack the glass or bear paramagnetic impurities difficult to remove (e.g., chromic mixture).
15. The extracts should be clear, no solid waste with a homogeneous volume.
16. Before NMR measurements the extracts should be placed at room temperature at least half an hour in order to avoid bad shimming owing to the temperature difference in the samples.
17. Before loading the NMR tube into the spectrometer, clean it with a tissue in order to take out grease from your hands.

18. Always clean new NMR tubes before the first use as they may have grease or impurities.
19. Use a suitable buffer, as $KH_2PO_4$, because the pH of the extracts can have an influence on the chemical shifts of compounds containing acid and basic groups.
20. Interactions with metal ions, hydrogen bonding, and other intermolecular interactions can also cause chemical shift displacements.
21. The chemical shifts of some metabolites can be changed by pH or their concentration (e.g., fumaric acid, citric acid, or malic acid).
22. By visual inspection of an NMR spectrum from Cannabis material, signals of amino acids (δ0.5–2.0), organic acids (δ2.0–3.0), sugars (δ3.0–5.0), and aromatic compounds (δ5–10) from methanol/water fractions, and signals of terpenoids and steroids (δ0.5–3.0), fatty acids (δ0.8–1.9 and δ5.08–5.4), and cannabinoids (δ4.3–8.16) from chloroform fractions can be identified.
23. For identification of metabolites compare chemical shift, kind of peak, and *J* value with those from reported reference compounds or your own NMR spectra database. Be careful that the values were obtained under the same conditions previously reported.
24. According to our conditions, the following metabolites can be identified from Cannabis cell cultures and plants based on chemical shift (δ), kind of peak, and coupling constant (*J*, Hz) from $^1$H-NMR spectra (21–24).

    In MeOD:$KH_2PO_4$ Buffer

    Adenosine δ6.04 (H-1′, *d*, *J*=6.6), δ8.23 (H-8, *s*), δ8.35 (H-2, *s*).

    Alanine δ1.48 (H-β, *d*, *J*=7.2), δ3.73 (H-α, *q*, *J*=7.2).

    Asparagine δ2.87 (H-3b, *dd*, *J*=16.9, 7.6), δ2.96 (H-3a, *dd*, *J*=16.9, 4.3), δ4.01 (H-2, *dd*, *J*=7.6, 4.3).

    Aspartic acid δ2.83 (H-β, *dd*, *J*=17, 7.9), δ2.94 (H-β′, *dd*, *J*=17, 4.0), δ3.95 (H-α, *dd*, *J*=8.1, 4.0).

    γ-aminobutyric acid δ1.90 (H-3, *m*, *J*=7.5), δ2.31 (H-2, *t*, *J*=7.5), δ3.00 (H-4, *t*, *J*=7.5).

    Choline δ3.21 (H-1′, H-2′, H-3′, *s*).

    Cytidine δ5.86 (H-5, *d*, *J*=8.0), δ5.91 (H-1′, *d*, *J*=4.3), δ7.93 (H-6, *d*, *J*=8.0).

    Ethanol glucoside δ1.24 (H-2, *t*, *J*=6.9).

    Fumaric acid δ6.54 (H-2, H-3, *s*).

    α-Glucose δ5.19 (H-1, *d*, *J*=3.8), δ5.24 (H-1, *d*, *J*=3.7).

    β-Glucose δ4.58 (H-1, *d*, *J*=7.9), δ4.64 (*d*, *J*=8.0).

Glutamic acid δ2.05 (H-β, *m*), δ2.36 (H-γ, *m*).

Glutamine δ2.13 (H-β, *m*), δ2.49 (H-γ, *m*).

Isoleucine δ0.95 (H-5, *t*, *J*=7.5), δ1.02 (H-6, *d*, *J*=6.8).

Leucine δ0.97 (H-5, *d*, *J*=6.7), δ0.98 (H-6, *d*, *J*=6.7).

Phenylalanine δ3.09 (H-3, *dd*, *J*=14.4, 8.4), δ3.30 (H-3′, *dd*, *J*=14.4, 9.6), δ3.94 (H-2), δ7.36 (H-5, H-6, H-7, H-8, H-9, *m*).

Sucrose δ4.19 (H-1′, *d*, *J*=8.5), δ5.40, 5.42 (H-1, *d*, *J*=3.8).

Threonine δ1.33 (H-γ, *d*, *J*=6.5), δ3.52 (H-α, *d*, 4.9), δ4.24 (H-β, *m*).

Tryptophan δ3.27 (H-3), δ3.50 (H-3′), δ3.98 (H-2), δ7.14 (H-8, *t*, *J*=7.7), δ7.22 (H-7, *t*, *J*=7.7), δ7.29 (H-11, *s*), δ7.47 (H-9, *dt*, *J*=8.0, 1.3), δ7.72 (H-6, *dt*, *J*=8.0, 1.3).

Tyramine δ6.85 (H-3, H-5, *d*, *J*=8.4), δ7.20 (H-2, H-6, *d*, *J*=8.4).

Tyramine glycoside δ7.11 (H-3, H-5, *d*, *J*=8.4), δ7.30 (H-2, H-6, *d*, *J*=8.4).

Tyrosine δ3.01 (H-β), δ3.20 (H-β′), δ3.86 (H-α), δ6.85 (H-3, H-5, *d*, *J*=8.4), δ7.18 (H-2, H-6, *d*, *J*=8.4).

Tyrosol δ6.80 (H-3, H-5, *d*, *J*=8.4), δ7.11 (H-2, H-6, *d*, *J*=8.4).

Trigonelline δ8.86 (H-4, H-6, *m*), δ9.15 (H-2, *s*).

Valine δ1.00 (H-γ, *d*, *J*=7.0), δ1.05 (H-γ′, *d*, *J*=7.0).

In $CD_3OD$

Phenylalanine δ3.14 (H-3, *dd*, *J*=15.9, 8.9), δ3.86 (H-2, *dd*, *J*=8.0, 4.0), δ7.03 (H-7, *t*, *J*=8.0), δ7.18 (H-11, *s*), δ7.35 (H-9, *d*, *J*=8.0), δ7.68 (H-6, *d*, *J*=8.0).

Gentisic acid δ6.61 (H-3, *d*, *J*=8.2), δ6.99 (H-4, *dd*, *J*=8.2, 2.5), δ7.21 (H-6, *d*, 2.5).

Glutamyl-tyramine δ2.05 (H-3″, *m*), δ2.38 (H-4″, *t*, *J*=7.2), δ3.56 (H-2″, *dd*, 15.0, *J*=7.2), δ3.34 (H-2′, *t*, *J*=8.0), δ2.68 (H-1′, *t*, *J*=8.0), δ6.69 (H-3, *d*, *J*=8.0), δ7.01 (H-2, *d*, *J*=8.0).

Tryptophan δ3.07 (H-3, *dd*, *J*=15.3, 8.0), δ3.91 (H-2, *dd*, *J*=8.0, 4.0), δ7.31 (H-5, *dd*, *J*=8.4, 1.6), δ7.39 (H-6, *t*, *J*=8.4).

Tyramine δ6.69 (H-3, H-5, *d*, *J*=8.4), δ6.78 (H-2, H-6, *d*, *J*=8.4).

Tyrosine δ6.62 (H-3, H-5, *d*, *J*=8.4), δ6.80 (H-2, H-6, *d*, *J*=8.4).

Tyrosol δ6.64 (H-2, H-6, *d*, 8.4), δ6.80 (H-2, H-6, *d*, *J*=8.4).

In $CDCl_3$

$\Delta^9$-Tetrahydrocannabinolic acid ($\Delta^9$-THCA) δ0.90 (H-5′, *t*, *J*=6.9), δ2.49 (H-1′, *m*), δ3.23 (H-10a, *dm*, *J*=7.0), δ6.25 (H-4, *s*), δ6.39 (H-10, *s*), δ12.19 (OH, *s*).

Cannabidiolic acid (CBDA) δ0.89 (H-5″, *t*, *J*=6.9), δ2.10, 2.20 (H-4, *m*), δ4.09 (H-1, *m*), δ5.56 (H-2, *s*), 6.26 (H-5′, *s*), δ6.63 (6′-OH, *s*), δ11.93 (2′-OH, *s*).

$\Delta^9$-Tetrahydrocannabinol ($\Delta^9$-THC) δ0.87 (H-5′, *t*, *J*=7.0), δ2.42 (H-1′, *m*, *J*=7.3, 1.6), δ3.20 (H-10a, *dm*, *J*=10.9), δ4.87 (OH, *s*), δ6.14 (H-2, *d*, *J*=1.6), δ6.26 (H-4, *d*, *J*=1.6), δ6.30 (H-10, *s*).

$\Delta^8$-Tetrahydrocannabinol ($\Delta^8$-THC) δ0.88 (H-5′, *t*, *J*=7.1), δ2.44 (H-1′, *td*, *J*=8.3, 2.1), δ2.70 (H-10a, *td*, 10.8, 4.8), δ3.24 (H-10, *dd*, *J*=16.5, 3.7), δ6.27 (H-4, *d*, *J*=1.5).

Cannabinol (CBN) δ0.89 (H-5′, *t*, *J*=6.8), δ2.5 (H-1′, *t*, *J*=7.5), δ5.13 (OH, *s*), δ6.29 (H-2, *d*, *J*=1.1) δ6.43 (H-4, *d*, *J*=1.1), δ7.07 (H-8, *d*, *J*=7.9), δ7.14 (H-7, *d*, *J*=7.9), δ8.16 (H-10, *s*).

Cannabidiol (CBD) δ0.88 (H-5″, *t*, *J*=6.8), δ1.55 (H-2′, *q*, *J*=7.6), δ2.43 (H-1″, *t*, *J*=7.5), δ3.90 (H-1, *m*, *J*=11.8), δ5.02 (6′-OH, *s*), δ5.57 (H-2, *s*), δ5.99 (2′-OH, *s*), δ6.26 (H-3′, *brs*).

Cannabigerol (CBG) δ0.90 (H-5″, *t*, *J*=6.9), δ1.56 (H-2″, *q*, *J*=7.8), δ2.45 (H-1″, *t*, *J*=7.5), δ3.41 (H-1′, *d*, *J*=7.0), δ5.07 (H-6′, *m*), δ5.29 (H-2′, *m*), δ5.36 (OH, *s*), δ6.0 (H-6, *s*), δ6.26 (H-4, *s*).

In $(CD_3)_2CO$ (Acetone-$d_6$)

Cannflavin A δ1.54 (H-8″, *s*), δ1.78 (H-9″, *s*), δ1.93 (H-4″, *t*, *J*=10.0), δ2.03 (H-5″, *t*, *J*=7.21), δ3.99 (OMe, *s*), δ5.04 (H-6″, *t*, *J*=7.08), δ7.02 (H-5′, *d*, 8.28), δ7.61 (H-2′, *d*, 1.88), δ13.30 (5-OH, *s*).

Cannflavin B δ1.80 (H-4″, *s*), δ3.36 (H-1″, *d*, *J*=7.12), δ3.99 (OMe, *s*), δ5.29 (H-2″, *tt*, *J*=7.24, 1.52), δ7.00 (H-5′, *d*, *J*=8.28), δ7.60 (H-2′, *d*, *J*=1.88), δ13.30 (5-OH, *s*).

25. For identification of the cannaflavins A and B purification steps by CC over HP-20 resin, silica gel, and Sephadex LH-20 are required.
26. A relative concentration of the intensities from target compounds can also be calculated, where a 100% value is assigned to control samples and increments or decrements are calculated in treated-samples.
27. Insufficient relaxation time gives an underestimation on the concentration of the compounds in the sample.

28. After an NMR measurement, the samples can be used for further analyses by column chromatography (Sephadex LH-20 column chromatography), HPLC or LC–MS. A cannabinoid profiling from chloroform fractions or flavonoid profiling from methanol:water fractions can be obtained.
29. For calibration of the spectrum, the internal standard or the solvent signal can be used.

## References


1. Flores-Sanchez IJ, Verpoorte R (2008) Secondary metabolism in cannabis. Phytochem Rev 7: 615–639
2. Radwan MM, Ross SA, Slade D, et al (2008) Isolation and characterization of new cannabis constituents from a high potency variety. Planta Med 74: 267–272
3. Radwan MM, ElSohly MA, Slade D, et al (2008) Non-cannabinoid constituents from a high potency *Cannabis sativa* variety. Phytochemistry 69: 2627–2633
4. Debruyne D, Moulin M, Bigot Mc et al (1981) Identification and differentiation of resinous cannabinoid textile Cannabis: combined use of HPLC and high-resolution GLC. Bull Narc 33: 49–58
5. Ross SA, ElSohly HN, Elkashoury EA, et al (1996) Fatty acids of Cannabis seeds. Phytochem Anal 7: 279–283
6. Hazekamp A, Simons R, Peltenburg-Looman A, et al (2004) Preparative isolation of cannabinoids from *Cannabis sativa* by centrifugal partition chromatography. J Liq Chromatogr Relat Technol 27: 2421–2439
7. Raharjo TJ, Verpoorte R (2004) Methods for the analysis of cannabinoid in biological materials: a review. Phytochem Anal 15: 79–94
8. Choi YH, Hazekamp A, Peltenburg-Looman A, et al (2004) NMR assignments of the major cannabinoids and cannabiflavonoids isolated from flowers of *Cannabis sativa*. Phytochem Anal 15: 345–354
9. Ross SA, ElSohly MA, Sultana GNN, et al (2005) Flavonoid glycosides and cannabinoids from the pollen of *Cannabis sativa* L. Phytochem Anal 16: 45–48
10. Hazekamp A, Peltenburg-Looman A, Verpoorte R, et al (2005) Chromatographic and spectroscopic data of cannabinoids from *Cannabis sativa* L. J Liq Chromatogr Relat Technol 28: 2361–2382
11. Gutierrez A, Rodriguez IM, Del Rio JC (2006) Chemical characterization of lignin and lipid fractions in industrial hemp bast fibers used for manufacturing high-quality paper pulps. J Agric Food Chem 54: 2138–2144
12. Flores-Sanchez IJ, Verpoorte R (2008) PKS activities and biosynthesis of cannabinoids and flavonoids in *Cannabis sativa* L. plants. Plant Cell Physiol 49: 1767–1782
13. Holmes E, Tang H, Wang Y et al (2006) The assessment of plant metabolite profiles by NMR-based methodologies. Planta Med 72: 771–785
14. Ward JL, Beale MH NMR spectroscopy in plant metabolomics. In Saito K, Dixon RA, Willmitzer L (ed) (2006) Plant Metabolomics, Biotechnology in Agriculture and Forestry, Vol. 57. Springer-Verlag, Berlin Heidelberg
15. Fiehn O (2002) Metabolomics: The link between genotypes and phenotypes. Plant Mol Biol 48: 155–171
16. Sumner LW, Mendes P, Dixon RA (2003) Plant metabolomics: large-scale phytochemistry in the functional genomics era. Phytochemistry 62: 817–836
17. Rochfort S (2005) Metabolomics reviewed: A new "omic" platform technology for systems biology and implications for natural products research. J Nat Prod 68: 1813–1820
18. Kim HK, Verpoorte R (2010) Sample preparation for plant metabolomics. Phytochem Anal 21: 4–13
19. Kim HK, Choi YH, Verpoorte R (2010) NMR-based metabolomic analysis of plants. Nat Protoc 5: 536–549
20. Ludwing C, Viant MR (2010) Two-dimensional *J*-resolved NMR spectroscopy: Review of a key methodology in the metabolomics toolbox. Phytochem Anal 21: 22–32
21. Choi YH, Kim HK, Hazekamp A, et al (2004) Metabolomic differentiation of *Cannabis*

*sativa* cultivars using $^{1}$H-NMR spectroscopy and principal component analyses. J Nat Prod 67: 953–957

22. Flores-Sanchez IJ, Pec J, Fei J, et al. (2009) Elicitation studies in cell suspension cultures of *Cannabis sativa* L. J Biotechnol 143: 157–168
23. Pec J, Flores-Sanchez IJ, Choi YH et al (2010) Metabolic analysis of elicited cell suspension cultures of *Cannabis sativa* L. by $^{1}$H-NMR spectroscopy. Biotechnol Lett 32: 935–941
24. Choi YH, Hazekamp A, Peltenburg-Looman AMG, et al. (2004) NMR assignments of the major cannabinoids isolated from flowers of *Cannabis sativa*. Phytochem Anal 15: 354–354