Chapter 4

Time-dependent metabolomics and transcriptional analysis of cannabinoid biosynthesis in *Cannabis sativa* var. Bedrobinol and Bediol grown under standardized condition and with genetic homogeneity

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Abstract

*Cannabis sativa* L. is mostly grown under non-standardized conditions resulting in high fluctuations of cannabinoid contents. The exact measure of these metabolites is hampered as most of the research has been conducted on seized, extracted or non-standardized cultivated *C. sativa* L. material. In this report legal standardized cultivation of two *C. sativa* L. chemotypes is investigated by HPLC-UV, qPCR and $^1$H-NMR, respectively during two cultivation phases of 4 weeks each. Based on HPLC the cannabinoid spectrum over 4 week cultivation period is monitored under specific light conditions and in correlation with the expression profile of *thca* and *cbda* genes in both chemotypes. Cannabinoid accumulation is observed specifically after shortening of the light period. Gene transcript based chemotyping was only possible comparing the *thca* and *cbda* transcription level ratio at the middle of the cultivation period, while at the end differences in gene transcription ratio diminished. $^1$H-NMR results indicate a clear difference between both chemotypes and cultivation phases by applying pattern recognition software.

The differences detected by using simple and non-destructive $^1$H-NMR were used to build a model, which can be applied to chemotyping and cultivation phase separation using selective $^1$H-NMR signals.
Introduction

*Cannabis sativa* L. is usually an annual, dioecious, herbaceous plant, even though records of monoecious *C. sativa* L. do exist [140, 141]. For over a thousand years, *C. sativa* L. is considered a multifunctional plant and, historically, it is used for the production of fiber and nutritional oils [142-145]. It is suggested to be one of the oldest cultivars grown by mankind [146]. More recently, the plant has been used and studied for medicinal purposes. Due to abuse of the plant for recreational purposes, cultivation of *C. sativa* L. varieties has been exclusively allowed to licensed cultivators.

Among the 109 cannabinoids known [59, 60], the biosynthetic pathway has only been elucidated for the major cannabinoids. In Figure 1 the biosynthesis of cannabigerolic acid (CBGA, 1.3), cannabidiolic acid (CBDA, 1.4), Δ⁹-tetrahydrocannabinolic acid (Δ⁹-THCA, 1.6) and cannabichromenic acid (CBCA, 1.5) from the precursors geranyl diphosphate (GPP, 1.1) and olivetolic acid (OA, 1.2) is depicted [85, 94, 105, 110, 112, 114, 147-149]. The four major cannabinoids (CBGA, CBDA, CBCA and Δ⁹-THCA) are naturally produced in their acidic form and represent the dominant fraction of cannabinoids within *C. sativa* L. Generally, CBDA and Δ⁹-THCA occur at higher concentrations than the others. Based on these two metabolites, a chemotype separation for legal and agricultural purposes has been proposed by the United Nations Office of Drugs and Crime (UNODC). Chemotype class I contains typically plants with a high content of Δ⁹-THCA and a low content of CBDA, and is considered the drug-type (ratio >>1). Chemotype class III encompasses plants with high amounts of CBDA and a relatively low amount of Δ⁹-THCA (fiber-type plants, ratio <<1), commonly referred to as hemp. Chemotype class II consists of plants with a near to equal amount of Δ⁹-THCA and CBDA (ratio between 0.5-2) [141].

Recently, several groups worldwide have evaluated the pharmacological activity of the cannabinoids present in all three chemotypes demonstrating their medicinal value. Whereas metabolites isolated from class I and II (high content of Δ⁹-THCA), are effective in pain and spasm reduction in multiple sclerosis patients [150, 151], metabolites from chemotype III plants (high content of CBDA) display anxiolytic and antipsychotic effects [152-154]. Even though proof on individual cannabinoids has been provided, the symmetric effect provided by the usage of plant material (combined effect of all metabolites) seems to reduce most of the side effects obtained with individual cannabinoids [155, 156].

Due to the pharmacological activities a large civilian movement is rising aiming to help decriminalizing the medicinal use of *C. sativa* L. in an increasing number of countries over
the world. Within The Netherlands, the medicinal use of *C. sativa* L. has been approved by the government thereby, allowing its consumption upon prescription. Since 2005 the Ministry of Health, Welfare and Sport has created an office for medicinal cannabis [157], which appointed one single company for the controlled cultivation of *C. sativa* L. [73]. Their strictly controlled environment offers the perfect conditions to study cannabinoid production within a genetically homogeneous plant population. Standardized parameters enhance the reproducibility of the results in independent experiments allowing an excellent comparison within *C. sativa* L. varieties. In this study, two *C. sativa* L. chemotype classes (I and II), originated from clonal individuals, were chosen for analyses. This report investigates the establishment of chemotype differences between these varieties during cultivation using $^1$H-NMR metabolomics and cbda / thca gene transcription profiles. Additionally, HPLC-UV analyses were performed to further confirm $^1$H-NMR data and to compare our results with previously reported chemotype differences.

![Figure 1](image_url)  

*Figure 1:* Biosynthesis of the major cannabinoids found within *C. sativa* L. according to [94]. (a) Cannabigerolic acid synthase (GOT) [105], (b) THCA synthase [85, 110, 127, 148, 149], (c) CBDA synthase [114] and (d) CBCA synthase [112]. The numbers below the compounds can be found within the second paragraph of the introduction.
Results and Discussion

Metabolome analysis for different chemotype varieties:

$^1$H-NMR is intensively employed to discriminate between plant varieties on the basis of metabolites [158-161]. Here $^1$H-NMR was applied to examine metabolic differences between cultivation phase and chemotype on clones prepared from $C$. sativa var. Bedrobinol (chemotype I) and var. Bediol (chemotype II). Each cultivation phase is characterized by a difference in hours of light exposure. The first phase (week 1-4) represents long days (18h of light), while the second (week 5-8) reflects short days (12h of light). To explore the potential of $^1$H-NMR to discriminate between the different chemotypes, a Principal Component Analysis (PCA) was performed. Identification of interesting signals occurred by using the orthogonal partial least squares (OPLS) analysis. Prior to analysis, raw data were corrected for the internal control (anthracene), followed by bucketing of the data and subsequently scaled according to the Pareto procedure [162, 163].

The complete PCA model consisted in a total of seven principle compartments (PCs), with the first three explaining 91.3 % of the variation within the data. The predictability of the model, set by the goodness of the fit, was found to be reliable (87.1 %) when obtained for the first three compartments. Visual analysis of the scatter plot (Figure 2A) revealed that PC1 and PC2 alone were responsible for the differentiation between the two chemotypes and the two cultivation phases. In particular, PC1 was responsible for the separation of cultivation phases whereas PC2 was responsible for the chemotype separation. The two compartments (PC1 and PC2) explained 85.2 % of the variation within the data, with a predictability of 82.3 % demonstrating the reliability of the model.

A more detailed analysis using a supervised method of pattern recognition based on the Orthogonal Partial Least Squares (OPLS) algorithm (Figure 2B), indicated buckets responsible for the discrimination between both, chemotypes and cultivation phases. The first model was produced using $Y =$ chemotype. This model explained a total of 94.2 % of the variation within the data and 98.3 % of variation within $Y$. The model had a total predictability of 96.9 %, confirming the reliability of the model. Even though the model could explain 94.2 % of the variation within the data, only 15.2 % was correlated to the chemotype. Four orthogonal compartments explained the remaining 79.1 %. The first compartment (66.1 %) is most likely explained by the separation between cultivation phases within the individual $C$. sativa L. chemotype (Figure 2B). With the help of S-plot analysis (Figure 2C) the buckets with the highest magnitude and reliability were selected and compared to the data from the
loading plot. This indicated that bucket 4.4, 4.44, 1.48 and 1.44 could be correlated with separation based on chemotypes, bucket 1.48 exhibiting the best separation profile (Figure 2D).

The second model based on the OPLS algorithm for Y = cultivation phase explained 66.4 % of the variation within the data, and up to 89.4 % of the variation of Y (data not shown). The predictability was 88.9 % and thus reliable. Additionally, the cultivation phase (Y) could be correlated to 66.4 % of the variation within the data. This observation confirms the suggestion of cultivation phase being responsible for the orthogonal variation found within the first OPLS model (Y = Chemotype). Subsequently, based on the S-plot, two buckets (1.64 and 0.88) could be identified (Figure 2E), which were responsible for the separation between cultivation phases. Bucket 0.88 provided the best separation profile in this model (Figure 2F).

A recent report described chemotype discrimination by comparing dried flower buds after completing cultivation [160], but no report has been published yet analyzing C. sativa L. growth over the complete cultivation period using $^1$H-NMR. Various primary metabolites, such as sugars and amino acids have previously been found to act as markers for chemotype differentiation [160]. Our studies employed an extended analytical set-up, allowing separation of both chemotypes and cultivation phases during the cultivation process. In addition to the previous report, we indicated that growth phases could be separated either according to the total amount of cannabinoids or to increasing amounts of individual cannabinoids. Bucket 0.88 included the hydrogen of the methyl group of the olivetolic acid moiety within cannabinoids, similar to previous reports on a new cannabinoid and on Δ$^9$-THCA and CBDA [160, 164]. The separation for chemotypes seems to specifically rely on the presence of CBDA, as the bucket identified overlaps with the peak corresponding to CBDA (bucket 1.48) confirming the results of a previous publication [160]. We could not directly relate primary metabolites to either the cultivation phase or chemotype. A possible explanation can be the type of extraction used. Our NMR samples were based on a small portion of the HPLC extracts (section 3.2) and therefore less concentrated when compared to the extraction procedures reported previous [160, 161, 165]. Consequently, some sensitivity in primary metabolite detection is diminished, but clearly it was still possible to discriminate between both cultivation phase and chemotype.

To make this classification more predictive, additional studies are needed allowing the specific identification of the metabolites responsible for the bucket-based separation. Moreover, small variations observed within buckets and published chemical shifts could be a
consequence of the differences in pH or solvents used during analysis and should consequently be handled with caution in standardized procedures.

![Figure 2](image-url)

**Figure 2**: A) PCA plot for PC1 (X-axis) and PC2 (Y-axis). B) OPLS plot based on chemotype. C) S-plot obtained from OPLS model based on chemotype. D) Chemotype separation plot based on bucket 1.48. E) S-plot obtained from OPLS model based on cultivation phase. F) Cultivation phase separation plot based on bucket 0.88. Grey color represents cultivation phase I, while black represents cultivation phase II. Arrow represents direction from cultivation phase I to II per chemotype. I) Represents chemotype I (P05_ C. sativa L. var. Bedrobinol) cultivation phase I. II) Represents chemotype I (P05_ C. sativa L. var. Bedrobinol) cultivation phase II. III) Represents chemotype II (P04_ C. sativa L. var. Bediol) cultivation phase I. IV) Represents chemotype II (P04_ C. sativa L. var. Bediol) cultivation phase II.
Cannabinoid profiling of selected C. sativa L. chemotype varieties:
The cannabinoid profile was quantified by employing HPLC-UV. The profile was recorded during the cultivation period and for every sample individually. Although similar reports have been published recently [166, 167], a difference exists between cultivation conditions and genetic background of the analyzed plant varieties. Therefore it was needed to confirm both the cannabinoid profile and the chemotype development.

Figure 3 represents the accumulation of all measured cannabinoids within both chemotypes and during the complete cultivation (week 1-8). During the first cultivation phase (week 1-4) the total cannabinoid content exhibited low variation as indicated by the absence of significant differences within and between the two chemotypes. As predicted by the $^1$H-NMR model provided above, the total amount of cannabinoids increased within both chemotype varieties during the second cultivation phase. The variance however, was only significant when comparing the first and second cultivation phase (p<<0.001). The observed results reveal a higher cannabinoid content within the last weeks of cultivation and can be explained by the previously reported increase in glandular trichome during flower development [87, 168-171]. It has been known that cannabinoid accumulate within the secretory cavity of the glandular trichome, especially the capitate-stalked trichome, and therefore an increase in trichome leads to an increase in cannabinoids.

![Figure 3: Total measured cannabinoid accumulation over the complete cultivation period (per week). Grey bars represent the chemotype II C. sativa L. var. Bediol and black bars represent chemotype I C. sativa L. var. Bedrobinol. Percentage is calculated as mg total cannabinoid per 100 mg of dried C. sativa L. flower material and represents the sum of all measured cannabinoids per week. Error bars for HPLC data are given as standard deviation.](image)

The chemotype varieties were further analyzed to spot time-dependent differences on the pattern of individual cannabinoids. Only Δ²-THCA and CBDA (Figure 4A and B) were...
present in amounts above 1 %, the remaining cannabinoids were only detected at low percentages (< 1 %). The time-dependent accumulation of Δ⁹-THCA and CBDA serve as a significant indicator to distinguish among chemotype varieties [166, 172] and could be confirmed by our analysis (Figure 4A and B). Our analysis indicated a strong chemotype settlement during the complete cultivation period, even though the chemotype ratio did vary per week. For the chemotype I variety the ratio remained >>1 (189-859), but was the strongest during week four (Figure 5A). The chemotype II variety had lower variance over the complete cultivation and remained between 0.72 and 1.04 (Figure 5B) and thus well within the limits set for chemotype II [141]. The strong variability of the chemotype I variety is explained by the low amount of CBDA was detected, during week 4 the CBDA level was the lowest amount from all weeks while the Δ⁸-THCA levels started to raise.

Similar to the major products CBDA and Δ⁹-THCA, the content of the precursor CBGA (data not shown) was determined. Interestingly, the amount of CBGA did not alter significantly during cultivation. In the second and third week a lower amount of CBGA were detected, increasing slowly over the fourth and fifth week, and stabilized finally. Overall, our analysis suggests a continuous presence of CBGA, further characterized by only a small variability within (p>0.05) and no significant differences between chemotype varieties analyzed here and reported elsewhere [166, 167].

Analysis of the neutral cannabinoids Δ⁹-THC and CBD was also performed. In the Bedrobinol variety, Δ⁹-THC was found during the first week of cultivation however, higher levels were present during the second cultivation phase. In this phase of cultivation, Δ⁹-THC was also detected for the Bediol variety. This cannabinoid reached a maximum of 0.5 % in both chemotypes, while CBD never exceeded 0.18 % for both varieties. The neutral cannabinoid CBG was only found in the Bediol chemotype during the second cultivation phase and remained below 0.05 %. The Δ⁹-THC derived metabolite, Δ⁸-THC, was only detected within the two final weeks of the second phase for both varieties and remained below 0.03 %. No CBN could be detected in any of the two chemotype varieties analyzed at any time.

HPLC analysis indicated no differences between the observed and reported [166] time dependent cannabinoid profiles. Although the chemotype ratio varied during the cultivation period for both analyzed varieties, both remained within the limits set by UNODC [141]. The results based on the time dependent profile, as provided by HPLC-UV, where consistent with the models provided by ¹H-NMR for both chemotype and cultivation phase separation.
Transcript analysis:

The analysis described in above, indicated that total cannabinoids, for the analyzed varieties, are dominated by both Δ^9-THCA and CBDA content. Consequently, these metabolites are used in chemotyping of the *C. sativa* L. varieties [141]. Previously, it has been reported that *C. sativa* L. chemotype profiles are dependent on the genomic Bt / Bd loci [124, 140, 173], representing the *thca* and *cbda* gene location, respectively. Here we report the transcript analysis of these two loci for the selected chemotype varieties during the complete cultivation.

Gene transcription varied tremendously between the weeks of cultivation, as shown in **Figure 4A and 4B**. Overall, gene transcription of *cbda* was lower than that of the *thca* gene, a surprising fact taking the chemotype differences obtained by HPLC-UV and ^1^H-NMR into consideration. Although a very low relative transcription levels of the *cbda* gene was predicted for chemotype I, a near to equal transcription level was expected for the chemotype II variety. Transcription of *cbda* in chemotype I was both lower than *cbda* transcription in chemotype II and than *thca* transcription levels in chemotype I. Nevertheless within both varieties the gene transcription profiles followed the same course, but differ between the two varieties. These observations indicate the presence of both the Bt and the Bd loci, and suggest that the chemotype dependent gene transcription for both *thca* and *cbda* is controlled by transcriptional regulation for, at least, the analyzed varieties.

In a recent report, a similar analysis was conducted [174]. Here the authors analyzed *thca* transcription levels in *C. sativa* L. flower material after cultivation ended. No chemotype differences were observed even when comparing to various endogenous controls (i.e. chalcone synthase, rubisco and 26S rRNA) [174]. Our analysis provides the possibility to analyze the transcription ratio per week. By obtaining the relative transcription between *thca* and gene location, respectively. Here we report the transcript analysis of these two loci for the selected chemotype varieties during the complete cultivation.

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cbda (ΔΔCt value), it could be concluded that chemotype differences during the last weeks of cultivation are indeed not significant. A tremendous increase in the relative transcription ratio was observed directly after changing the hours of light exposure (Figure 5A and B). Although it had been long known for C. sativa L. that accumulation of cannabinoids increases when shortening the hours of light exposure, this is the first time that it is shown to affect directly transcription level ratios when comparing cbda and thca transcription levels. More interestingly, when comparing the relative transcription and the Δ9-THCA / CBDA metabolite ratio for chemotype I (Figure 5A), both peaked at week 4, while for chemotype II (Figure 5B) the transcription ratio peaked one week later and no significant increase could be observed for the metabolite ratio.

Besides the obvious metabolic differences, these results clearly indicate differences in gene transcription regulation between the selected chemotype varieties. Interestingly the difference for relative transcription ratio between C. sativa L. varieties remained >10 fold higher during the first cultivation phase and the first week of the second cultivation phase, although differences in transcription ratios decreased during the last three weeks of cultivation (<4.8 fold). The results indicate that chemotyping by gene transcription is, at least for thca and cbda gene transcripts, time-dependent and can be detected at best when light exposure simulates long days for the varieties analyzed within this report.

**Conclusions**

Using genetic analysis and 1H-NMR metabolome analysis, two bio-analytical methods have been studied and adopted to discriminate between chemotype varieties over the growth cycle. Moreover, it was shown that differences in content and pattern of THCA/CBDA and other minor cannabinoids were significant in the early and late phase of the growing processes subsequently presenting important markers to follow up during biosynthesis in standardized cultivations. These differences were also evident for mRNA transcripts of genes encoding for Δ9-THCA and CBDA synthases, respectively, when the hours of light exposure were reduced. Moreover, 1H-NMR showed a fast, sensitive and non-destructive method for chemotype differentiation during the complete cultivation period. Although both HPLC and 1H-NMR were not able to classify the samples according to the week of cultivation, a clear separation was observed between both, the chemotype and the cultivation phase.

The results provided in this paper present promising methodologies to discriminate in detail among the various chemotype varieties and growth phases. Furthermore, evidence is provided
Figure 5: Ratio analysis of the metabolites ($\Delta^9$-THCA/CBDA) and the relative transcription between cbda and thca ($2^{\Delta\Delta Ct}$ shown per C. sativa L. variety. Y-axis (right) in logarithmic scale). A) Ratio analysis of chemotype I C. sativa L. var. Bedrobinol. B) Ratio analysis of chemotype II C. sativa L. var. Bediol. No detectable transcription ratio could be obtained for the chemotype I C. sativa L. var. Bedrobinol in week 2 and 3 due to an absence of cbda transcripts. Error bars are defined by upper and lower limits for gene transcription and by SD for metabolites.

for difference in transcriptional regulation between the analyzed chemotypes with different genotype backgrounds. By changing light exposure hours, it is suggested that transcriptional regulation is responsible for the differences occurring between chemotype varieties. Besides the direct applicability of the reported methods by governmental institutions, our results provide insights into the role of transcriptional gene regulation on the chemotype differences.

Material and methods

Chemicals and Reagents:
Cannabinoid reference compounds for delta-9-tetrahydrocannabinol ($\Delta^9$-THC), delta-8-tetrahydrocannabinol ($\Delta^8$-THC), cannabigerol (CBG), cannabidiol (CBD), cannabichromene (CBC), cannabinol (CBN), cannabinolic acid (CBNA), $\Delta$-9-tetrahydrocannabinolic acid ($\Delta^9$-THCA), cannabigerolic acid (CBGA) and cannabidiolic acid (CBDA) were purchased from Prisna BV (Leiden, The Netherlands). All chemicals were purchased at Merck Biosolve Ltd (Valkenswaard, The Netherlands) except for anthracene (Sigma Aldrich).
Plant origin, cultivation and sampling:

*Cannabis flos* var. Bediol® (P04_2008_1-24) and *Cannabis flos* var. Bedrobinol® (P05_2008_1-24) were cultivated on Rockwool at Bedrocan BV (Veendam, The Netherlands), under standardized conditions without using pesticides or heavy metals. All plants grown were at the same room and exposed to equal conditions. All plants were exposed to High Pressure Sodium (HPS) light and given an average of 20,550 lm/m². When lights were on, relative humidity was kept between 35-60 %, whereas without light, relative humidity increased to 45-80 %. Humidity and temperature were controlled by an automated climate control system. An automated watering system, employing a top dripping mechanism was used to supply water and basic fertilizing nutrients to the plants. Clean air was used to provide movement to the plants; the climate was tightly controlled. Air was taken from the outdoor environment and filtered before being brought inside the cultivation room.

A standardized process of cutting rooting and stress recovery was applied for all plants, followed by 4 weeks (Cultivation phase I) of long day (18h day, 6h night) and subsequently 4 weeks (Cultivation phase II) of short day simulation (12h day and night). Only cultivation phase II and I were used for analysis. Sampling occurred once a week for over 8 weeks. Plants were randomly chosen within the plant cultivation room and taken from the fourth branch from the top. During cultivation phase I the young developing flowers were separated and directly frozen in liquid nitrogen. During cultivation phase II, the flower buds were separated in a similar manner as described for cultivation phase I. Flower development was scored according to a previous report [175], flower during cultivation phase I scored 1-4 [175]. Plant material was stored at -80°C until analysis.

Sample extraction for HPLC-UV and NMR analysis:

Plant specimens were crushed and homogenized in liquid nitrogen using a pestle and a mortar. The pestle and the mortar were rinsed thoroughly between each sample. Samples were divided for metabolite extraction and RNA extraction. Samples for RNA extraction were stored at -80°C until further processing whereas samples for metabolite extraction were exposed to 24h of freeze drying, allowing a reduction of 78.23 % ± 1.48 % (W/W). For metabolite extractions about 100 mg of plant specimen was taken and 5 ml of HPLC grade methanol was added. Samples were exposed to the following treatment for four times: Methanol exposed samples were incubated for 10 min at room temperature under slight shaking, followed by 10 min in a sonication bath (Branson 5210 Ultrasonic Bath, Gemini BV, The Netherlands) and short centrifugation to collect plant specimen at the bottom of the tube.
Methanol fractions were pooled for each sample in a volumetric flask and the total volume adjusted to 25 mL after addition of 1 mg of anthracene as internal control. Extracts were homogenized by shaking and stored at -20 °C. Before analysis extracts were allowed to warm up to room temperature and filtered using a syringe filter (0.22 µm). The first 8 mL of the filtered flow-through were discarded and only the last ± 2 mL used for HPLC analysis. Samples were kept in the dark during the whole procedure.

**HPLC-UV analysis:**

HPLC analysis was performed using a Shimadzu-VP system (Shimadzu, ‘s-Hertogenbosch, The Netherlands) consisting of a LC-10ATvp pump, a Kontron 360 auto sampler, a SPD-M10Avp DAD detector, a DGU-20A5 degasser, a FCV-10ALvp low pressure gradient mixer, a SCL-10Avp system controller, a FIAtrom system CH-30 column heater (USA) and CLASS-VP software, Version 6.12SP4. For separation a Gemini-NX C18 (4.6x150 mm, 3 µm, Phenomenex) column combined with an Agilent Technologies Guard column C18 (4.6x12.5 mm, 5 µm) was used. An injection volume of 25 µL was used for all samples. When the cannabinoid area under the curve (AUC) exceeded the linear range, set by external calibration curves, injection was repeated using a smaller volume (5 µL). The mobile phase consisted of Milli-Q (5% acetonitrile, phase A) and 95% acetonitrile (phase B), both acidified with 0.1 % trifluoroacetic acid. The employed method consisted of 50 % phase B, increasing linearly over 40 min to 60 % phase B. Thereafter, the gradient increased from 60 % to 80 % of phase B in 10 min. The method ended with an equilibration step that returned the parameters to the starting conditions within 2 min, and was maintained for 10 min before the next injection. All peaks were analyzed at 225 nm and column temperature was kept constant at 35°C.

**1H-NMR analysis:**

For 1H-NMR analysis, 10 mL of extract was dried under a stream of nitrogen while keeping samples on ice. The total weight was determined before suspension of the samples in 1 mL of MeOH-d4. 1H-NMR spectra were recorded at 25°C on a 500 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. MeOH-d4 was used as the internal lock. Each 1H-NMR spectrum consisted of 128 scans requiring 10 min and 26 sec acquisition time using the following parameters: 0.16 Hz/point, pulse width (PW) = 30° (11.3 ms), and relaxation delay (RD) = 1.5 sec. A presaturation sequence was used to suppress the residual H2O signal with low power selective irradiation at the H2O frequency during the recycling delay. FIDs were Fourier transformed
with LB = 0.3 Hz. The resulting spectra were manually phased and baseline corrected, and calibrated using anthracene at 8.43 ppm (s), using R statistical software (version 2.14.0).

**RNA extraction and qPCR:**
Samples were taken from -80°C and kept in liquid nitrogen. Before RNA extraction, plant specimens were ground to a fine powder using a pestle and a mortar. During the procedure the material was kept cold using liquid nitrogen. Fresh weights were registered (± 80 mg per sample) for the sample prior RNA extraction according to the manufacturer’s instructions (Macherey-Nagel GmbH & Co. KG, Düren, Germany). RNA concentration, purity and integrity was determined and only proper samples were analyzed by qPCR using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands) as described in the iScript kit manual. Primers used were selected on the basis of efficiency and correlation coefficient by diluting pooled RNA from all weeks and plant types tested. Primer sequences, efficiency, slope and correlation coefficient can be found in **Table 1**. Transcription levels were corrected and relativized against the S18 housekeeping gene using the ΔCt and ΔΔCt method [176].

*Table 1: Primers for qPCR used within this study, including their efficiency, slope and correlation coefficient.*

<table>
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<tr>
<th>Name</th>
<th>Fw sequence 5’-3’</th>
<th>Bw sequence 5’-3’</th>
<th>Efficiency (%)</th>
<th>R²</th>
<th>Slope</th>
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<td>P13THCA</td>
<td>GAACCTTGTTTA</td>
<td>GTGTAATATTAGG</td>
<td>108.5</td>
<td>0.953</td>
<td>-3.134</td>
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<td></td>
<td>CACTGCCTCC</td>
<td>ACTCGCATG (1528)</td>
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<tr>
<td>P2CBDA</td>
<td>GGAAGAATAAG</td>
<td>TAACCTTAATCTTGA</td>
<td>100.3</td>
<td>0.940</td>
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<td>AAGCACCAG(1150)</td>
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<td></td>
<td>CAC (910)</td>
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<td>P5S18</td>
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<td></td>
<td>(1298)</td>
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**Metabolomics software and statistical analysis:**
Anthracene normalized HPLC samples were statically analyzed using R software (Version 2.14.0) and ANOVA analysis based on mg of cannabinoid per mg of plant specimen (DW). Differences associated with p-values < 0.001 were considered significant for HPLC data. ¹H-NMR data was calibrated for the anthracene singlet at δ=8.43 and bucketed into 0.01 and 0.04
ppm buckets. The area within 3-4 ppm was deleted due to the presence of the solvent peak and other highly overlapping peaks. Data with chemical shifts bigger than 8.45 ppm was discarded. Data was analyzed using Principal Component Analysis (PCA) and Orthogonal Partial Least Squares (OPLS) algorithms using the SIMCA-P+ software (version 12.0.1). The OPLS analysis was based on chemotype (Y1) and cultivation phase (Y2). Confidence limits were set using Hotelling T2 (0.95) procedure according to SIMCA-P+ procedures.
“It is during our darkest moments that we must focus to see the light.”

- Aristotle Onassis