Monitoring Metabolite Profiles of *Cannabis sativa* L. Trichomes during Flowering Period Using ^1H NMR-Based Metabolomics and Real-Time PCR

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Key words

- *Cannabis sativa*
- Cannabaceae
- trichomes
- Metabolomics
- RT-PCR
- cannabinoid

Abstract

Cannabis sativa trichomes are glandular structures predominantly responsible for the biosynthesis of cannabinoids, the biologically active compounds unique to this plant. To the best of our knowledge, most metabolomic works on *C. sativa* that have been reported previously focused their investigations on the flowers and leaves of this plant. In this study, ^1H NMR-based metabolomics and real-time PCR analysis were applied for monitoring the metabolite profiles of *C. sativa* trichomes, variety Bediol, during the last 4 weeks of the flowering period. Partial least squares discriminant analysis models successfully classified metabolites of the trichomes based on the harvest time. Δ^9 -Tetrahydrocannabinolic acid (1) and cannabidiolic acid (2) constituted the vital differential components of the organic preparations, while asparagine, glutamine, fructose, and glucose proved to be their water-extracted counterparts. According to RT-PCR analysis, gene expression levels of olivetol synthase and olivetolic acid cyclase influenced the accumulation of can-

nabinoids in the *Cannabis* trichomes during the monitoring time. Moreover, quantitative ^1H NMR and RT-PCR analysis of the *Cannabis* trichomes suggested that the gene regulation of cannabinoid biosynthesis in the *C. sativa* variety Bediol is unique when compared with other *C. sativa* varieties.

Abbreviations

- CBCA (3): cannabichromenic acid
- CBD (5): cannabidiol
- CBDA (2): cannabidiolic acid
- CBDAS: cannabidiolic acid synthase
- OAC: olivetolic acid cyclase
- OLS: olivetol synthase
- PLSDA: partial least squares discriminant analysis
- THC (4): Δ^9 -tetrahydrocannabinol
- THCA (1): Δ^9 -tetrahydrocannabinolic acid
- THCAS: Δ^9 -tetrahydrocannabinolic acid synthase
- TMS: tetramethylsilane

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Introduction

Trichomes are small protrusions of epidermal origin found on the surfaces of leaves and other organs of many plants [1]. One of the valuable compound classes produced in the trichomes are cannabinoids. These compounds are found only in *Cannabis sativa* L. (Cannabaceae), a prospecting medicinal plant. THC, a decarboxylated form of THCA, is the most studied cannabinoid. This compound is a well-established psychoactive component of *Cannabis* [2] that activates cannabinoid receptors CB1 and CB2 [3, 4].

As an annual plant, *C. sativa* has two growth periods: vegetative and flowering. In the former, the plant is growing rapidly and producing only low

amounts of the relevant metabolites, whereas the flowering stage is the period of accelerated cannabinoid biosynthesis. According to our previous report, cannabinoid production in the *Cannabis* flower increases with the time of blooming, and reaches the highest level during the last few weeks of the stage [5]. However, the details of cannabinoid production in the *Cannabis* trichomes during blooming are still unclear. Some metabolomics works on *C. sativa* have been reported previously. GC together with partial component analysis (PCA) has been successfully applied for chemotaxonomic discrimination of *Cannabis* varieties and quality control of plant materials [6]. ^1H NMR coupled with PCA has been used for differentiation of *Cannabis* cultivars as

well [7]. In addition, ^1H NMR-based metabolomics along with RT-PCR has been performed for investigating cannabinoid production of *Cannabis* cultivars during the flowering period [5]. Moreover, metabolomic data of *Cannabis* from multiple tissues is now available at a database of medicinal plant metabolomics (<http://metnetdb.org/PMR/>) [8]. However, the previous reports focused their metabolomics studies on the flowers and leaves of *Cannabis*. The intention of this report is to stress metabolomics analysis of the Bediol variety in direct comparison and to clarify the biosynthetic development of a comprehensive cannabinoid pattern for the same time and conditions. Therefore, application of metabolomics for investigating cannabinoids from trichomes, including influencing primary metabolites, may lead to a better understanding of the cannabinome. In this study, we report on *Cannabis* trichome metabolomics in order to monitor metabolite profiles, especially cannabinoids, and determine the differential compounds during the last 4 weeks of the flowering period. Our metabolomic investigation was carried out using the ^1H NMR technique together with PLSDA. Furthermore, we also applied RT-PCR for investigating the expression levels of cannabinoid genes during the monitoring time.

Results and Discussion

In order to identify metabolites in the *C. sativa* trichomes of the variety Bediol, all collected samples were grouped into two fractions, namely chloroform and water extracts, and then analyzed using an NMR method. ^1H NMR spectra of the chloroform samples were investigated for the identification of cannabinoids. As a result, three acidic and two neutral cannabinoids were successfully identified, including THCA (1) and its neutral form THC (4), CBDA (2) and its neutral form CBD (5), and CBCA (3). Resonances belonging to the protons of THCA (1) and CBDA (2) were clearly distinguishable in the ^1H NMR spectra, as shown in **Fig. 1**, and thus point to both as the predominant components of the chloroform extracts of the *Cannabis* trichomes. Signals correspondence to CBCA (3) were confirmed based on comparison with published data [9]. Meanwhile, assigned signals of other cannabinoids in the ^1H NMR spectra were further confirmed by analysis of 2D NMR spectra (not shown) and compared with the data of reference cannabinoids. Detail ^1H NMR data of the identified cannabinoids can be found in **Table 1**, while their chemical structures are depicted in **Fig. 2**.

Concentrations of the identified cannabinoids in the trichome samples were successfully determined using quantitative ^1H NMR spectroscopy, as seen in **Fig. 3**. THCA (1) and CBDA (2) were detected at high concentrations and dominated other cannabinoids. As an example, the concentration of THCA (1) in the *Cannabis* trichomes at week 8 was 25.29 mg/g of fresh trichomes weight. Other cannabinoids were recorded in low concentrations, as depicted in **Fig. 3**.

According to the quantitative analysis by ^1H NMR, all concentrations of analyzed cannabinoids increased significantly in weeks 5 and 6. As known from previous our own work [5], this biosynthetic cannabinoid peak was confirmed again. In the following last two weeks (weeks 7 and 8), concentrations of THCA (1) and CBDA (2) did not vary too much and only increased slightly. As a consequence, during the late cultivation period, THCA (1), CBDA (2), and CBCA (3) biosynthesis remained static. In parallel, decarboxylation occurred significantly as shown by the increasing concentration of THC (4) from week 6 to week 7. Later, THC (4) con-

Fig. 1 Characteristics signals of cannabinoids identified in the ^1H NMR spectra of the chloroform extracts of *C. sativa* trichomes, variety Bediol.

centrations dropped in the last two weeks of the flowering period, maybe due to further degradation or release as a volatile compound. Meanwhile, the concentration of the decarboxylated CBD (5) increased during the monitoring time.

Investigation of ^1H NMR spectra obtained from water extracts revealed the presence of amino acids and carbohydrates in the *Cannabis* trichomes (**Table 1**). The fingerprint signals of alanine, asparagine, glutamine, glutamic acid, glycine, leucine, proline, threonine, and valine were detected within the δ 0.8–4.0 ppm region of the spectra. Meanwhile, in the δ 4.0–6.0 ppm region, the anomeric signals of α -glucose, β -glucose, β -mannose, fructose, and sucrose were recorded. Moreover, a sugar alcohol, inositol, was found in the samples as well. Other organic compounds characteristic of the investigated aqueous extracts included acetic acid, formic acid, fumaric acid, succinic acid, and choline. All the distinguishing signals of the identified water-soluble compounds were confirmed with the literature [7, 10–16] and can be found in **Table 1**. Moreover, the concentrations of alanine, asparagine, glutamine, glutamic acid, threonine, glucose, fructose, sucrose, inositol, and choline were determined semiquantitatively in all of the samples investigated within the monitoring period. The recorded production patterns of all of the quantified water-soluble compounds during the last 4 weeks of the flowering period are shown in **Fig. 4**.

Multivariate analysis (MVA) methods were applied for in-depth profiling of metabolite biosynthesis processes in the *Cannabis* trichomes over the last 4 weeks of their flowering period. Since the unsupervised multivariate method (principal component analysis) could not provide enough separations (data not shown), PLSDA (a supervised approach) was selected as the principal investigative procedure. It uses a discrete class matrix and is based

Table 1 Characteristic ^1H NMR chemical shifts of the identified compounds.

Compounds		Chemical shift (ppm) and coupling constants (Hz)
Chloroform fraction	CBCA (3)	δ 5.49 (H-7, d, J = 10.1), δ 6.23 (H-4, s), δ 6.74* (H-8, d, J = 10.1)
	CBDA (2)	δ 0.90 (H-5', m), δ 1.33 (H-3', m), δ 1.33 (H-4', m), δ 1.55 (H-2', m), δ 1.72 (H-12, s), δ 1.80 (H-11, s), δ 2.39 (H-6a, m), δ 2.79 (H-1a', m) and 2.92 (H-1b', m), δ 4.10 (H-10a, s), δ 4.40 (H-13cis, s), δ 4.55 (H-13trans, s), δ 5.57* (H-10, s), δ 6.26 (H-4, s), δ 11.85 (COOH, s)
	CBD (5)	δ 1.63 (H-12, s), δ 4.66* (H-13trans, brs), δ 6.18 (H-2, brs)
	THCA (1)	δ 0.90 (H-5', m), δ 1.12 (H-13, s), δ 1.33 (H-3', m), δ 1.33 (H-4', m), δ 1.45 (H-12, s), δ 1.55 (H-2', m), δ 1.69 (H-11, s), δ 2.79 (H-1a', m) and 2.92 (H-1b', m), δ 3.23 (H-10 a, brd, J = 10.9), δ 6.25 (H-4, s), and δ 6.39* (H-10, s), δ 12.20 (COOH, s)
	THC (4)	δ 6.13* (H-2, brs), δ 6.28 (H-4, s), δ 6.30 (H-10, brs)
Water fraction	α -Glucose	δ 5.23* (H-1, d, J = 3.8)
	β -Glucose	δ 4.64 (H-1, d, J = 7.9)
	β -Mannose	δ 4.99 (H-1, d, J = 7.9)
	Acetic acid	δ 1.91 (s)
	Alanine	δ 1.48* (H-3, d, J = 7.2)
	Asparagine	δ 2.87* (H-3b, dd, J = 16.9, 7.6), δ 2.96 (H-3a, dd, J = 16.9, 4.3), δ 4.01 (H2, dd, J = 16.9, 4.3)
	Choline	δ 3.21* (CH ₃ , s)
	Formic acid	δ 8.46* (s)
	Fructose	δ 4.06* (H-1, d, J = 3.5)
	Fumaric acid	δ 6.59 (s)
	Glutamic acid	δ 2.14 (H-4, m), δ 2.46* (H-3, m)
	Glutamine	δ 2.07* (H-4, m), δ 2.38 (H-3, m)
	Glycine	δ 3.58 (H-2, s)
	Inositol	δ 3.25 (H-5, t, J = 9.3), δ 3.49 (H-1, dd, J = 9.9, 2.9), δ 3.61* (H-4, t, J = 9.3)
	Leucine	δ 0.95 (H-5, d, J = 6.6), 0.97 (H-6, d, J = 6.6)
	Proline	δ 2.35 (H-3, m), δ 4.06 (H-2, m)
	Succinic acid	δ 2.45 (s)
	Sucrose	δ 4.22* (H-1' of fructose moiety, d, J = 8.6), δ 5.42 (H-1, d, J = 3.8)
	Threonine	δ 1.33* (H-5, d, J = 6.6)
	Valine	δ 1.00 (H-3, d, J = 6.8), δ 1.05* (H-4, d, J = 6.8)

* Signal is used for the quantification analysis.

on the partial least squares (PLS) model in which the dependent variable is chosen to represent class membership [17]. The PLSDA model of the chloroform extracts of the *Cannabis* trichomes successfully discriminated the samples based on their harvest time. This model possessed four PLS components, and explained 69.9% and 88.4% of the total variations (R^2X and R^2Y , respectively), and 71.9% of the predictive ability (Q^2). The score plot of PLS1 (29.8%) and PLS2 (14.4%) resulted in a clear definition of four well-separated clusters corresponding to their harvest time (see **Fig. 5A**). A subsequent investigation of the PLS1 loading plot indicated that THCA (1) and CBDA (2) were the crucial discriminant compounds as depicted in **Fig. 5B**. In the case of the water extracts of the trichomes in question, the obtained PLSDA model comprised six PLS components with R^2X = 77.2%, R^2Y = 96.4%, and Q^2 = 82.9%. A well-separated cluster was obtained in the score plots of PLS1 (41.2%) and PLS2 (12.9%), as documented in **Fig. 6A**. Responsible compounds for the classification were identified through the investigation of the PLS1 loading plot as shown in **Fig. 6B**. Valine, alanine, asparagine,

Fig. 3 Concentration of cannabinoids in the *Cannabis* trichomes, variety Bedil, during the last 4 weeks of the flowering period.

glutamic acid, sucrose, fructose, and choline were detected contributing to the discrimination of metabolite profiles of the water extracts.

Quantitative RT-PCR experiments were performed in order to investigate the expression level of cannabinoid genes in the *Cannabis* trichomes at weeks 5, 6, 7, and 8 of the flowering period. Cannabinoid biosynthesis is initiated by the formation of olivetolic acid, an important precursor of THCA (1) and CBDA (2). Olivetolic acid is a product of condensation of malonyl-CoA and hexanoyl-CoA by OLS [18] and OAC [19]. Based on RT-PCR analysis, the highest expression levels of OLS and OAC genes were found in the trichomes as depicted in **Fig. 7B**. Furthermore, gene ex-

Fig. 4 Concentration of quantified compounds in the water extracts of *Cannabis* trichomes, variety Bediol, over the last 4 weeks of the flowering period.

pression levels of OAC and OLS in the *Cannabis* trichomes indicated a declining pattern during the monitoring time, as shown in **Fig. 7A**. This might lead to a decrease in the biosynthesis of olivetolic acid with a reduced production of THCA (1) and CBDA (2) in the trichomes as a consequence. Interestingly, the declining pattern of these genes seems in accordance with the production of both compounds in the trichomes during the monitoring time as explained previously. Therefore, these results suggest that gene expression levels of OLS and OAC are in relation with the accumulation of THCA (1) and CBDA (2) on the metabolome level in the *Cannabis* trichomes over the monitoring time.

THCAS and CBDAS are responsible for the transformation of cannabigerolic acid, a product of condensation between olivetolic acid and geranyl pyrophosphate, into THCA (1) [20] and CBDA (2) [21], respectively. RT-PCR analysis revealed that THCAS and CBDAS genes were less expressed in the trichomes than in the *Cannabis* leaves, as seen in **Fig. 7B**. This finding was in accordance with our previous work [22]. Furthermore, gene expression levels of THCAS and CBDAS in the *Cannabis* trichomes were almost stable during the monitoring time, as depicted in **Fig. 7A**. These results might indicate that THCAS and CBDAS contributed less to the accumulation of THCA (1) and CBDA (2) during the last 4 weeks of the flowering period as expected.

Based on cannabinoid composition, generally, *Cannabis* is classified into two types, namely, medicinal and fiber plants. Normally, the medicinal plant contains a high concentration of THCA (1) or its neutral form THC (4), and a low amount of CBDA (2) or its neutral form CBD (5) [23–26]. Meanwhile, cannabinoid composition of the fiber plant is opposite that of the profile of medicinal *Cannabis* [23–26]. This probably indicates that THCA (1) competes with its isomer, CBDA (2), in the biosynthesis of the cannabinoids, as both are derived from the same precursor, cannabigerolic acid. *Cannabis* used in our research belongs to the variety Bediol and is considered medicinal *Cannabis* because of abundant THCA (1). However, this plant also contains high concentrations of CBDA (2), which is why it is of interest to have a closer look at the metabolomics profile. The cannabinoid profile of this variety differs between tissues and trichomes, but, in general, the trend of high THCA (1) and CBDA (2) concentrations in all tissues was confirmed. The quantitative analysis during the last 4 weeks of the flowering period also showed that the production of THCA (1) and CBDA (2) in the *Cannabis* trichomes had a similar pattern, as

depicted in **Fig. 3**. The same profile was also observed by analysis of the expression levels of the essential biosynthetic genes for both compounds. As depicted in **Fig. 7A**, the gene expression levels of THCAS and CBDAS in the *Cannabis* trichomes possessed a similar pattern over the monitoring time. Therefore, these results probably indicate a unique regulation on the cannabinoid biosynthesis in the *C. sativa* variety Bediol, and might be different from the fiber plant or other medicinal *Cannabis*.

In conclusion, biosynthetic pattern characteristics in trichomes of the variety Bediol over the final 4 weeks of the flowering period were investigated using ^1H NMR-based metabolomics and RT-PCR analysis. Special emphasis was put on metabolic profiling of biologically active cannabinoids and their relation to primary metabolites like monomeric sugars and amino acids. The metabolomics approach, relying on PLSDA modeling, afforded differentiation of the metabolite profiles of the *Cannabis* trichomes based on the harvest time. Moreover, quantitative ^1H NMR, together with RT-PCR analysis, has successfully revealed the pattern of cannabinoid biosynthesis in the *Cannabis* trichomes during the last weeks of flowering time. In this work, we showed for the first time in *C. sativa* that ^1H NMR-based metabolomics in combination with RT-PCR is a powerful tool that can be successfully applied for time-dependent monitoring of biosynthetic processes in the trichomes.

Materials and Methods

Reagents and chemicals

First-grade chloroform, methanol, deuterated chloroform (99.8%), deuterium oxide (99.8%), TMS, trimethylsilane propionic acid sodium salt (TSP), and sodium deuteroxide were purchased from Carl Roth GmbH. Anthracene was obtained from Sigma-Aldrich Chemie GmbH. Reference compounds of cannabinoids, CBDA (2), CBD (5), THCA (1), and THC (4) were purchased from THC Pharm GmbH and tested for purity and identity by NMR and LC-MS prior to use.

Plant material

Standardized *C. sativa*, variety Bediol (THC level approx. 6%; CBD level approx. 7.5%), was supplied by Bedrocan BV. The plants were grown indoors under standardized conditions as explained in the previous report [27]. Briefly, they were initially generated from cuttings of standardized mother plants and cultivated under controlled, long daylight conditions (18 h/day). After the vegetative growth phase, the flowering stage was induced under a shorter (12 h/day) light regime for 8 weeks. The trichomes were isolated and analyzed from week 5 to week 8 of the flowering period. Plant specimens were assigned voucher numbers (D5.26.06.2012, D6.02.07.2012, D7.09.07.2012, D8.16.07.2012) and deposited at the Technical Biochemistry Department, TU Dortmund. All plant handling and experimental procedures were carried out under the license No. 4584989, issued by the Federal Institute for Drugs and Medical Devices (BfArM), Germany.

Trichomes isolation

Cannabis trichomes in this research were isolated according to Yerger et al. [28] with slight modifications. Fresh flowers of *C. sativa*, variety Bediol, were put into liquid nitrogen. Floral leaves and the stigma were removed using forceps, with occasional resubmerging of the flowers in liquid nitrogen. Afterwards, a 5- to 10-g flower sample was transferred into a 50-mL falcon tube

Fig. 5 Multivariate data analysis computed for the chloroform extracts of *Cannabis* trichomes, variety Bediol. The PLSDA score plot (A) shows classifying the samples based on their harvest time. The loading plot (B) reveals the discriminant compounds in the differentiation, namely: THCA (1); CBDA (2); CBCA (3). The permutation test plot (C) for the PLSDA modeling. (Color figure available online only.)

Fig. 6 Multivariate data analysis computed for the water extracts of *Cannabis* trichomes, variety Bediol. The PLSDA score plot (A) shows classifying the samples based on their harvest time. The loading plot (B) reveals the discriminant compounds in the differentiation. The permutation test plot (C) for the PLSDA modeling. (Color figure available online only.)

and placed in liquid nitrogen. The tube was then removed from the liquid nitrogen tank and approximately 2 to 3 cm³ of finely powdered dry ice (prepared by wrapping a piece in clean paper towels and crushing with a pestle) were added. Immediately, the tube was loosely capped and vortexed at maximum speed for approximately 1 min, and then the flowers were removed. To obtain the trichomes, the content of each tube was sieved through a nylon net filter with a pore diameter of 140 µm (Merck Millipore) into a 500-mL glass beaker surrounded by dry ice. The trichomes were subsequently transferred into 2 mL frozen microcentrifuge tubes with a spatula; the samples were placed in liquid nitrogen. In this work, trichomes were isolated at 4 different harvest times

during the flowering period, namely, weeks 5, 6, 7, and 8. Six biological replicates of the *Cannabis* trichomes were collected at each harvest, thus, in total, twenty-four samples were prepared.

Extraction

Two hundred mg of fresh *C. sativa* trichomes were transferred into a centrifuge tube. Two mL of 50% aqueous methanol and 2 mL of chloroform were added to the tube followed by vortexing for 1 min and sonication for 1 min. Subsequently, the sample was shaken at 200 rpm for 1 h at 30°C. The water and chloroform phases were separated by pipetting and filtering into new centrifuge tubes. The chloroform fraction was dried in a rotary vacuum

Fig. 7 Expression levels of cannabinoid genes in the trichomes during the monitoring time (A) and in the different organs of *Cannabis* (B). L: Leaves; F: Flowers; T: Trichomes.

evaporator at 30 °C under pressure of 31 mbar. The water fraction was dried in a freeze-dryer. In total, 24 chloroform fractions and 24 water fractions were prepared.

NMR measurements

^1H NMR spectra of the samples were recorded using a Bruker Avance DRX 500 spectrometer (Bruker BioSpin GmbH) operating at 500.13 MHz. Dry chloroform extracts were dissolved in deuterated chloroform containing TMS as an internal standard and anthracene (1 mg/sample) as a quantitative internal standard. ^1H NMR spectra of the chloroform extracts were recorded with the following parameters: acquisition time = 5.23 s, relaxation delay = 5 s, pulse width = 3 μs , free induction decay (FID) data points = 64 K, spectral width = 12531.32 Hz, and number of scans = 128. Potassium dihydrogen phosphate was added to deuterium oxide as a buffering agent. The pH of the deuterium oxide was adjusted to 6.0 using a 1 N sodium deuteroxide solution. Afterward, the water extracts were dissolved in the deuterium oxide containing trimethylsilane propionic acid sodium salt (TSP, 0.01 %, w/v) as an internal standard. ^1H NMR spectra of the water extracts were recorded using a presaturation pulse program with the following parameters: acquisition time = 2.72 s, relaxation delay = 2 s, FID data points = 32 K, spectral width = 12019.23 Hz, number of scans = 128. Processing of FIDs was performed with line broadening set to 1.0 Hz using ACD/Labs 12.0 software. All ^1H NMR spectra were manually phased and baseline corrected.

PLSDA analysis

For the chloroform extracts, ^1H NMR spectra were scaled to TMS and then reduced to integrated regions of equal width (0.02 ppm), corresponding to the region of δ 0.50–13.00 ppm. The region δ 7.24–7.27 ppm was excluded from the analysis because of the residual signal of chloroform. The region of anthracene peaks, namely, δ 7.44–7.48 ppm, δ 7.96–8.06 ppm, and δ 8.42–8.48 ppm, was removed as well. ^1H NMR spectra of the

water extracts were scaled to TSP and reduced to integrated regions of equal width (0.02 ppm) within δ 0.50–10.00 ppm. The regions of δ 3.28–3.37 ppm and δ 4.50–5.00 ppm were removed from the analysis due to the residual signals of methanol and water. Bucketing was performed by ACD/Labs 12.0 software with scaling on total intensity. PLSDA was performed with SIMCA-P software (v. 13.0, Umetrics). PLSDA scaling was based on the Pareto method. Each type of the trichomes extracts were divided into four classes based on their harvest time (weeks 5, 6, 7, and 8) and then analyzed with PLSDA. The percent of the response variation explained by the PLSDA model (R^2X and R^2Y), and the percent of the response variation predicted by the model according to cross validation (Q^2) were calculated. Each model of PLSDA was validated with the permutation test applying 300 permutations. According to the obtained results (► **Figs. 5C** and **6C**), all Q^2 values of the permuted Y vectors were lower than the original ones and the regressions of the Q^2 lines intersected the y-axis at points below zero, thus confirming the validity of the applied PLSDA models.

Quantitative ^1H NMR

In order to quantitatively evaluate cannabinoid biosynthesis in the *Cannabis* trichomes over the last 4 weeks of the flowering period, the obtained ^1H NMR data were further processed according to a technical report [29]. The proton signals recorded for the chloroform extracts in the range of δ 2.0–7.0 ppm were selected for quantification to ascertain optimal discrimination of resonance patterns indicating the best selectivity. Anthracene was used as an internal standard since it has a simple ^1H NMR spectrum consisting of a singlet (δ 8.43) and two quartets (δ 8.01, δ 7.48), and does not overlap with the signals of cannabinoids. The quantification was conducted by calculating the relative ratio of the peak area of selected proton signals of the target cannabinoids to the singlet peak of anthracene. The relative quantification of the metabolites identified in the water extracts was performed by measuring the ^1H NMR signal area of the corresponding signals and comparing them to the TSP signal. Analysis of variance (ANOVA) for relevant ^1H NMR signals was performed using Microsoft Excel 2010.

RNA isolation and real-time PCR

In this research, the expression levels of cannabinoid genes, namely, THCAS, CBDAS, OLS, and OAC, in the *Cannabis* trichomes were investigated at four different harvest times (weeks 5, 6, 7, and 8) using RT-PCR. In addition, we also carried out analysis of the expression levels of these genes in different *Cannabis* organs, namely, the leaves, flowers, and trichomes, at week 7 of the flowering period. Fresh *Cannabis* materials (the isolated trichomes, flowers, and leaves) were ground to a fine powder using a pestle and mortar under cold conditions (with adding liquid nitrogen). Afterwards, around 90 mg of fresh fine powder of the plant materials were prepared for RNA isolation using an RNeasy Plant Mini Kit (Qiagen). The isolation procedure followed the manufacturer's instructions. Concentration of isolated RNA was determined with a Qubit RNA Assay Kit (Life Technologies). Equivalent quantities of RNA isolated from the trichomes were reverse transcribed using an iScript cDNA synthesis kit (BIO-RAD Laboratories) according to the manufacturer's instructions. Quantitative real-time PCR experiments were performed with Applied Biosystems StepOnePlus Real-Time PCR Systems (Applied Biosystem) and SYBR Green PCR Master Mix (Applied Biosystem). These experiments were carried out using a comparative CT method

Gene	Forward Primer	Reverse Primer
18S	GAGAAACGGCTACCATCA	CCGTGTCAGGATTGGTAATTT
THCAS	AAGTTGGCTTGCAGATTCGAA	TGTAGACATACCTCAGCATCA
CBDAS	AAAGTTGGCTTGCAGATTCGA	GGACATGCCCTCAGCATCA
OLS	GGGCTGCTGCGGTGATT	TATCGGCTTTCCCCAACT
OAC	TGTTGATTGGAGATGTCATCG	TTCGTGGTGTAGTCAAAATGA

Table 2 Primers of cannabinoids genes for RT-PCR analysis.

($\Delta\Delta CT$) [30]. The S18 housekeeping gene was used as an endogenous control gene. Gene sequences of THCAS [20], CBDAS [21], OLS [18], and OAC [19] were used as the templates for designing primers using Primers Express Software v.3.0.1 (Applied Biosystem). The list of primers can be seen in **Table 2**. RT-PCR was run with the following conditions: preincubation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and then the melt curve stage at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 min.

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Conflict of Interest

The authors declare no conflict of interest.

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