Metabolic Changes in the Trichomes of *Cannabis sativa* var. *bedrobinol* Analyzed by ^{1}H-NMR-Based Metabolomics

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Abstract: *Trichomes of* Cannabis sativa *are the main tissue for synthesizing and storing cannabinoids, the most interesting compounds in this plant. In this report, metabolic changes in the trichomes of* C. sativa *var.* bedrobinol *were investigated by* ^{1}*H-NMR-based metabolomics over the flowering session. Three cannabinoids, including* Δ^9*-tetrahydrocannabinolic acid (THCA), cannabichromenic acid (CBCA), and* Δ^9*-tetrahydrocannabinol (THC), were successfully identified in the chloroform extracts of the* Cannabis *trichomes. Meanwhile, 20 non-cannabinoid compounds, including sugars, amino acids, and other acidic constituents, were detected in the water extracts. Metabolic changes of the* Cannabis *trichomes during the monitoring time were successfully revealed using the models of partial least squares discriminant analysis (PLSDA) and* ^{1}*H-NMR quantitative analysis. Score plots of the PLSDA models classified metabolomes based on the harvest time. Discriminant metabolites for the differentiation were detected in the loading plots of the models. THCA was found as an important discriminant compound in the chloroform extracts, while all quantified water-soluble compounds were detected, contributing to the metabolic changes of the water extracts. The obtained results shed more light on the biosynthesis of metabolites in the* Cannabis *trichomes over the flowering season.*

Keywords: Cannabis*; cannabinoids;* ^{1}*H-NMR-based metabolomics; trichomes*

■ INTRODUCTION

Cannabis sativa L. (Cannabaceae) is an annual flowering plant that has been cultivated for thousands of years ago. The *Cannabis* cultivation method was written in ancient Chinese literature, and this method had been used for a thousand years [1]. The plant has been used by humankind for medicine, food, clothing, and purposes of recreation and spiritual [2-3]. This plant originally comes from Central and Eastern Asia region [4] and then spread around the world, including Indonesia. Since *Cannabis* is often misused as a recreational drug, this plant is categorized as a dangerous narcotic in most countries, including Indonesia. However, some drugs derive from *Cannabis* are available for medication, including Marinol® and Sativex®. Marinol® is used for the treatments of HIV/AIDS-induced anorexia and chemotherapy-induced nausea and vomiting [5]. Meanwhile, Sativex® can be used for the treatment of neurologic disorders, including multiple sclerosis [6].

The responsible compounds for bioactivities of *Cannabis* are cannabinoids. More than 100 cannabinoids had been successfully detected in *Cannabis* [7]. Δ^9-tetrahydrocannabinol (THC) is the most interesting cannabinoid since it is mainly responsible for the psychoactive properties of *Cannabis*. This cannabinoid presents in the plant on its acidic form (Δ^9-tetrahydrocannabinolic acid, THCA). Based on the concentrations of THC and cannabidiol (CBD), *Cannabis* can be categorized in 3 types, namely THC type (THC >> CBD), CBD type (THC << CBD) and intermediate type (THC ≈ CBD). Cannabinoids are predominantly synthesized and deposited in the

trichomes of *Cannabis*. At least there are 3 types of trichomes in *Cannabis*, including capitate-stalked, capitate-sessile, and bulbous trichomes. Based on our previous works, capitate-stalked trichomes contain more cannabinoids compare to the others [8].

Metabolomics as one of the emerging omics tools has been applied for studying *Cannabis*. Gas chromatography-based metabolomics had been applied to classify *Cannabis* cultivars based on their varieties [9]. This method had been also used to discriminate *Cannabis sativa* from *Cannabis indica* [10]. HPLC-based metabolomics had successfully been employed to study the impact of domestication on *Cannabis* metabolomes [11]. The same method had been applied to study the terpene profiles in different *Cannabis* cultivars [12]. HPLC-ESIHRMS/MS based-metabolomics was used to evaluate the chemical composition of *Cannabis* medicinal extracts [13]. ^{1}H-NMR based-metabolomics as one of the advance metabolomic methods was employed in *Cannabis* study as well. This method had been successfully used to differentiate *Cannabis* cultivars [14]. Moreover, ^{1}H-NMR based-metabolomics had been applied to study the impact of elicitation in cell suspension cultures of *Cannabis* [15-16]. Our previous work also applied ^{1}H-NMR-based metabolomics that combined with real time PCR (RT-PCR) technique for studying cannabinoid biosynthesis of *Cannabis* cultivars over flowering season [17]. This combined method was used to differentiate *Cannabis* metabolome based on their organs [18]. Recently, we used the same approach for monitoring cannabinoid biosynthesis in the trichomes of *Cannabis* during the flowering period [19].

The sample used in our previous work [19] was *C. sativa* var. *bediol*, intermediate type *Cannabis* containing almost equal concentrations of THC and CBD. It elucidated cannabinoid biosynthesis in the trichomes of intermediate type *Cannabis*. However, to the best of our knowledge, the changes in metabolite profiles in THC-type *Cannabis* during the flowering period is still unclear. Therefore, in continuation of our studies on this plant, here we report ^{1}H-NMR-based metabolomics in the trichomes of *C. sativa* var. *bedrobinol*. This variety is THC type *Cannabis* containing high THC and almost zero CBD. The purpose of this report is to study metabolic change and to differentiate metabolomes of trichomes of *C. sativa* var. *bedrobinol* over the last flowering weeks.

■ EXPERIMENTAL SECTION

Materials

The standardized *C. sativa* var. *bedrobinol* was obtained from Bedrocan BV (Veendam, Netherlands). Chloroform and methanol used in the extraction were obtained from Carl Roth GmbH (Karlsruhe, Germany). Tetramethylsilane (TMS), trimethylsilane propionic acid sodium salt (TSP), sodium deuteroxide, deuterated chloroform, and deuterated water that required for the NMR measurement were bought from Carl Roth GmbH (Karlsruhe, Germany). Anthracene, as the quantitative standard compound, was purchased from Sigma-Aldrich GmbH (Darmstadt, Germany). Reference THCA and THC were obtained from THC Pharm GmbH (Frankfurt, Germany).

Instrumentation

An Eppendorf 5415D centrifuge (Eppendorf AG, Hamburg, Germany) was used to centrifuge the samples. A Buchi rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) was applied to remove the solvents on the sample extracts. A Bruker Avance DRX 500 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) was used to record ^{1}H-NMR spectra of the *Cannabis* trichomes.

Procedure

Trichomes isolation

All plant handling and experiment procedures in this research were performed based on license No. 4584989 issued by the Federal Institute for Drugs and Medical Devices (BfArM), Germany. All experiments were carried out in the Laboratory of Technical Biochemistry, Technical University of Dortmund, Germany. The trichomes of *C. sativa* var. *bedrobinol* were isolated based on previous reports [19-20]. The floral leaves and the stigma of the fresh *Cannabis* flowers were removed using forceps. A 5–10 g of the flower was moved into a 50 mL centrifuge tube and placed for a while in a tank containing liquid nitrogen. A finely

powdered dry ice was added into the tube. Immediately, the tube was loosely capped and vortexed for 1 min. During the vortex process, the trichomes were released from the *Cannabis* flower. Afterward, the flower was removed from the tube. The trichomes were collected by filtering other materials from the tube with a 140 μm nylon net filter (Merck Millipore). In this work, the *Cannabis* trichomes were isolated at 4 different weeks over the flowering season, including weeks 5, 6, 7 and 8.

Extraction

The fresh trichomes of *C. sativa* var. *bedrobinol* in a centrifuge tube (200 mg) was extracted by 2 mL of water-methanol solvent (1:1) and 2 mL of chloroform. The tube was vortexed for 1 min and sonicated for 1 min. Afterward, the sample was incubated at a shaker machine (200 rpm) for 1 h at 30 °C. The water phase was separated from the chloroform phase by pipetting. The chloroform fraction was separated from the trichomes by filtering. The chloroform fraction was dried by a rotary evaporator at 30 °C and 31 mbar. Meanwhile, the water fraction was dried in a freeze-drying machine.

NMR measurements

Dry chloroform extracts of *Cannabis* trichomes were dissolved in deuterated chloroform. Tetramethylsilane (TMS) was used as an internal standard, while anthracene (1 mg/sample) was used as a quantitative standard compound. 128 scans of 64 K data points are recorded with a spectral width of 12531.32 Hz, the acquisition time of 5.23 sec and a relaxation delay of 5 sec. Dried water extracts of *Cannabis* trichomes were dissolved in deuterated water containing phosphate buffer (pH 6) and trimethylsilane propionic acid sodium salt (TSP, 0.01%, w/v) as an internal standard. ^{1}H-NMR spectra of the water extracts were recorded using a presaturation method. 128 scans of 32 K data points are recorded with a spectral width of 12019.23 Hz, the acquisition time of 2.72 sec and a relaxation delay of 2 sec. The free-induction decay (FID) NMR data were processed with ACD/Labs 12.0 software (Advanced Chemistry Development, Inc., Toronto, Canada). This software was also used for referencing, phasing, baseline correction of the ^{1}H-NMR spectra.

Quantitative analysis

^{1}H-NMR quantitative analysis was applied to determine concentrations of some identified metabolites semi-quantitatively. The identified cannabinoid was quantified based on a previous report [21]. The concentration of cannabinoids was determined by comparing the signal of the targeted cannabinoids with the singlet signal of anthracene (δ 8.45 ppm). Meanwhile, the quantification of the identified metabolites in the water extracts was carried out by comparing the corresponding signal area to the TSP signal.

Multivariate data analysis

Alignment and bucketing of the ^{1}H-NMR spectra were performed using ACD/Labs 12.0 software (Advanced Chemistry Development, Inc., Toronto, Canada). ^{1}H-NMR spectra of the chloroform extracts were scaled to TMS. Bucketing was carried out by integrating regions of equal width (0.02 ppm) within δ 0.50–13.00 ppm and performed with an intelligent bucketing option. The region δ 7.24–7.27 ppm was removed from the analysis because of the chloroform signal. The region of anthracene signals (δ 7.44–7.48, 7.96–8.06, and 8.42–8.48 ppm) were excluded from the analysis. ^{1}H-NMR spectra of the aqueous extracts were scaled to TSP. Bucketing of these spectra was performed by integrating regions of equal width (0.02 ppm) from δ 0.50–10.00 ppm. The residual water signal at δ 4.73–5.22 ppm was excluded from the data analysis. The processed data sets extracted from the ^{1}H-NMR spectra were imported into SIMCA-P version 13.0 (Umetrics, Umeå, Sweden) for the multivariate statistical analysis. Partial Least Squares Discriminant Analysis (PLSDA) was applied as the primary method for extracting maximum separation among samples.

■ RESULTS AND DISCUSSION

Metabolite Identification

For identifying metabolites present in the trichomes of *C. sativa* var. *bedrobinol*, the ^{1}H-NMR spectra of both extracts were investigated. Δ^9-tetrahydrocannabinolic acid (THCA) and its neutral form, Δ^9-tetrahydrocannabinol

(THC), were successfully identified in the ^{1}H-NMR spectra of the chloroform extracts. Other identified cannabinoid in the spectra was cannabichromenic acid (CBCA). The proton signals belong to THCA were clearly distinguishable in the ^{1}H-NMR spectra, indicating this compound was a major metabolite in the *Cannabis* trichomes. Four methyl groups of THCA were detected in the spectra at δ 0.90 (H-5', m), 1.12 (H-13, s), 1.45 (H-12, s), and 1.69 ppm (H-11, s). Other aliphatic proton signals belong to THCA were identified at δ 1.33 (H-3', m), 1.33 (H-4', m), 1.55 (H-2', m), 2.79 (H-1a', m), 2.92 (H-1b', m) and 3.23 ppm (H-10a, brd, J = 10.9). Two aromatic protons of THCA were recorded at δ 6.25 (H-4, s) and δ 6.39 ppm (H-10, s). Meanwhile, one chelating proton signal belongs to the carboxyl group of THCA was detected at δ 12.20 ppm (COOH, s). THC and CBCA were identified by identifying their characteristic signals in the ^{1}H-NMR spectra of the chloroform extracts. However, the signals of THC and CBCA in the spectra were low, indicating both are minor compounds in the trichomes. The characteristic proton signals of THC were identified at δ 6.13 (H-2, brs), 6.28 (H-4, s) and 6.30 ppm (H-10, brs). Meanwhile, the signals of CBCA were recorded at δ 5.49 (H-7, d, J = 10.1), 6.23 (H-4, s) and 6.74 ppm (H-8, d, J = 10.1). The signals of CBCA were further verified by comparison with published data [22]. Proton signals of THCA and THC in the ^{1}H-NMR spectra were further verified by comparing the data with their reference spectra. Structures of identified cannabinoids are depicted in Fig. 1.

Investigation of ^{1}H-NMR spectra of the water extracts successfully identified amino acids, sugar compounds, and other organic compounds. The fingerprint signals of alanine (δ 1.48 ppm, 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 ppm, H-2, dd, J = 16.9, 4.3), glutamine (δ 2.07, H-4, m; δ 2.38 ppm H-3, m), glutamic acid (δ 2.14 H-4, m; δ 2.46 ppm, H-3, m), glycine (δ 3.58 ppm, H-2, s), leucine (δ 0.95, H-5, d, J = 6.6; δ 0.97 ppm, H-6, d, J = 6.6), proline (δ 2.35, H-3, m; δ 4.06 ppm, H-2, m), threonine (δ 1.33 ppm, H-5, d, J = 6.6) and valine (δ 1.00, H-3, d, J = 6.8; δ 1.05 ppm, H-4, d, J = 6.8) were successfully recorded in the spectra within the δ 0.8–4.0 ppm region. Meanwhile, in the δ 4.0–6.0 ppm region, the proton signals of α-glucose (δ 5.23 ppm, H-1, d, J = 3.8), β-glucose (δ 4.64 ppm, H-1, d, J = 7.9), β-mannose (δ 4.99 (H-1, d, J = 7.9), fructose (δ 4.06 ppm, H-1, d, J = 3.5), 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) and sucrose (δ 4.22, H-1', d, J = 8.6), δ 5.42 ppm, H-1, d, J = 3.8) were detected as well. Further investigation of the obtained ^{1}H-NMR spectra revealed other organic acids, including acetic acid (δ 1.91 ppm, s), formic acid (δ 8.46 ppm, s), fumaric acid (δ 6.59 ppm, s), succinic acid (δ 2.45 ppm, s) and choline (δ 3.21 ppm, s). All the fingerprint signals of the identified compounds were further verified with the data from reported works [14,23-25].

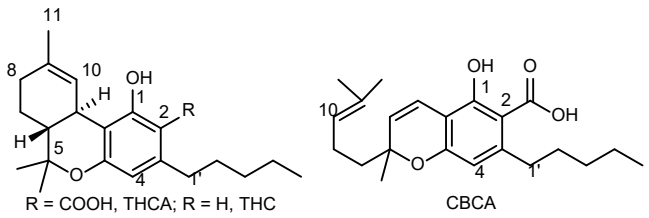

Fig 1. Chemical structures of identified cannabinoids

^{1}H-NMR Quantitative Analysis

The concentrations of some identified metabolites were determined semi-quantitatively by ^{1}H-NMR technique. According to the analysis of chloroform extracts of the trichomes of *C. sativa* var. *bedrobinol*, concentrations of THCA in weeks 5 and 6 did not vary too much as described in Table 1. Nevertheless, the concentrations of these compounds increased significantly in week 7 and enhanced slightly in the following week. Meanwhile, concentrations of CBCA and THC increased over the monitoring time.

Besides, as the main site for synthesizing secondary metabolites, trichomes are well known as the main place for storing secondary metabolites, including cannabinoids [26]. Although THCA and CBCA could be decarboxylated by heat into their neutral forms (THC and CBC) [27], however, both are the end products of cannabinoid biosynthesis [28-29]. Therefore, concentrations of cannabinoids that quantified in the ^{1}H-NMR analysis, were the accumulated concentrations of THCA, CBCA, and THC, which produced from the initial cannabinoid

biosynthesis till the harvesting. It was confirmed by the results of the quantitative analysis that showed the concentration enhancement of the cannabinoids over the flowering period as depicted in Table 1.

The amounts of alanine, asparagine, choline, fructose, glucose, glutamine, glutamic acid, inositol, sucrose, threonine, and valine in the water extracts of the trichomes of *C. sativa* var. *bedrobinol* were successfully determined. Their concentrations were diverse during the monitoring period. These quantified metabolites are primary metabolites that directly involved in the normal growth, development, and maintenance of the cellular functions of the plant. Besides that, these metabolites are also precursors or intake compounds in the biosynthesis of secondary metabolites. The diverse concentrations of the quantified compounds over monitoring time were probably correlated to their functions as primary metabolites. The recorded production patterns of the quantified metabolites over monitoring time are described in Table 1.

Multivariate Data Analysis

In this research, the extracted data of the ^{1}H-NMR spectra were further analyzed with multivariate data analysis for investigating metabolite profiles of the trichomes of *C. sativa var. bedrobinol* over the last flowering weeks. In the first step, the extracted data were treated with principal component analysis (PCA) method, an unsupervised pattern-recognition approach. However, this method could not give enough separations (data not shown); thus, the analysis was continued further with partial least square discriminant analysis (PLSDA) method, supervised pattern-recognition approach. This method applies a discrete class matrix and is based on the partial least squares (PLS) model [30].

PLSDA modeling of the chloroform extract data set resulted in the good separation of the trichomes of *C. sativa var. bedrobinol* based on their harvesting time. This PLSDA model had 6 components, 74.8% of cross validation coefficient (Q2), and explained 83.0% and 91.2% of total variations (R2X and R2Y, respectively). This model was further validated by the permutation test (300 permutations). The test produced the regressions of Q2 line that intersects the y-axis at points below zero [Q2 = (0.00, -0.0741); R2 = (0.00, 0.392)]. This result validated the PLSDA model statistically. The best separation model of the chloroform extracts was obtained when combining the first (34.7%) and the second (18.8%) PLSDA components. This score plot successfully illustrated metabolic discrimination of the trichomes of *C. sativa var. bedrobinol* based on their harvesting time,

Table 1. Concentrations of quantified compounds in the trichomes of *C. sativa* var. *bedrobinol*, over the last flowering weeks

Compound	Concentration (mg/g of fresh trichomes weight ± SD)			
	Week 5	Week 6	Week 7	Week 8
CBCA (δ 6.74)	0.60 ± 0.10	0.77 ±0.12	0.92 ± 0.30	1.12 ± 0.40
THCA (δ 6.39)	45.27 ± 2.29	45.90 ± 3.04	64.39 ± 6.39	67.65 ± 7.19
THC (δ 6.13)	0.09 ± 0.01	0.19 ± 0.08	0.38 ± 0.09	0.99 ± 0.06
Alanine (δ 1.48)	1.39 ± 0.19	1.42 ± 0.26	1.54 ± 0.38	1.66 ± 0.68
Asparagine (δ 2.87)	11.80 ± 1.74	13.66 ± 2.16	16.38 ± 2.16	15.30 ± 3.80
Choline (δ 3.21)	2.11 ± 0.15	2.22 ± 0.18	2.34 ± 0.41	1.65 ± 0.20
Fructose (δ 4.06)	10.33 ± 1.49	11.84 ± 0.63	11.97 ± 0.63	9.43 ± 0.35
Glucose (δ 5.23)	1.36 ± 0.22	1.57 ± 0.48	2.28 ± 0.84	1.28 ± 0.32
Glutamic acid (δ 2.46)	1.94 ± 0.25	2.08 ± 0.21	2.66 ±0.43	2.22 ± 0.58
Glutamine (δ 2.07)	1.49 ± 0.22	1.54 ± 0.31	1.33 ± 0.29	1.25 ± 0.19
Inositol (δ 3.61)	4.52 ± 0.49	5.45 ± 1.10	5.13 ± 0.64	5.44 ± 2. 47
Sucrose (δ 4.22)	8.75 ± 1.82	10.31 ± 1.65	10.72 ± 1.63	10.90 ± 2.88
Threonine (δ 1.33)	7.31 ± 1.29	4.56 ± 0.59	2.84 ± 0.59	3.84 ± 0.89
Valine (δ 1.05)	0.36 ± 0.07	0.52 ± 0.10	0.47 ± 0.14	0.43 ± 0.07

as depicted in Fig. 2(a). For investigating the responsible compounds in the classification, the loading plot of the first PLSDA component was analyzed. As described in Fig. 2(b), THCA was identified as the most discriminant compound in the classification of the metabolic trichomes based on their harvesting time.

In our previous work, we found that THCA and cannabidiolic acid (CBDA) were detected as important discriminant compounds in the classification of trichome metabolomes of *C. sativa* var. *bediol* during the flowering period [19]. *C. sativa* var. *bediol* is intermediate-type *Cannabis* containing almost equal concentrations of THC (a neutral form of THCA) and CBD (a neutral form of CBDA). Meanwhile, in this report, we detected THCA as the most discriminant compound in the classification. It was possibly due to the sample used in this report is categorized as THC-type *Cannabis* containing high THC and almost zero CBD. However, this result confirmed THCA as the crucial cannabinoid that synthesized in high amounts in the trichomes of *Cannabis* over flowering weeks and had an important role in the metabolome differentiation.

PLSDA model of the water extracts successfully classified the trichomes of *C. sativa var. bedrobinol* according to the collecting times. This model possessed 9 PLS with R2X = 62.0%, R2Y = 99.6%, and Q2 = 67.7%. The model validation was carried out by the permutation test (300) resulting the regressions of Q2 lines intersected the y-axis at points below zero [Q2 = (0.00, -0.399); R2 = (0.00, 0.301)]. The score plot consisting of the first component (13.1%) and the third component (5.1%) revealed four well-separated groups, as seen in Fig. 3(a). Based on the loading plot investigation of the first component (Fig. 3(b)), all quantified water-soluble

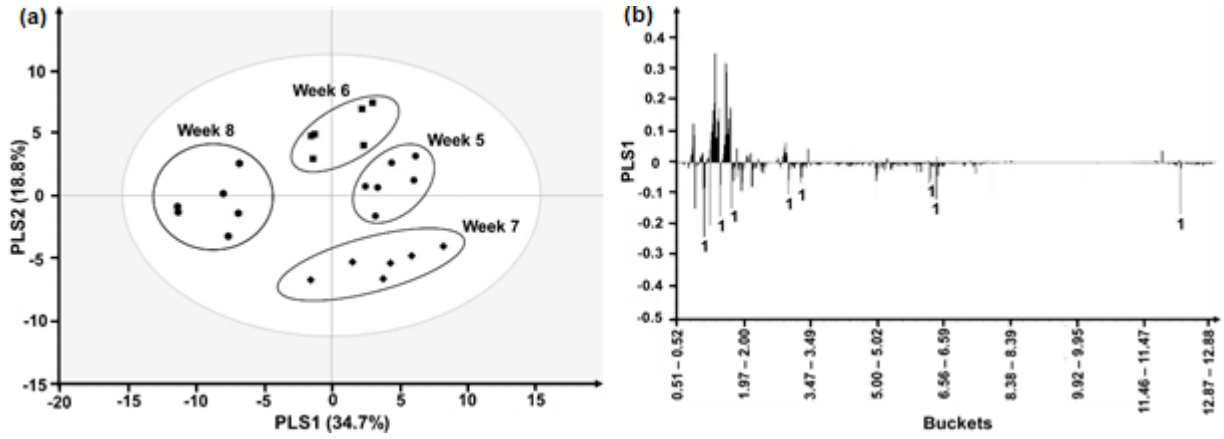

Fig 2. PLSDA score (a) and loading (b) plots from the ^{1}H-NMR spectra of chloroform extracts of *bedrobinol* trichomes 1: THCA

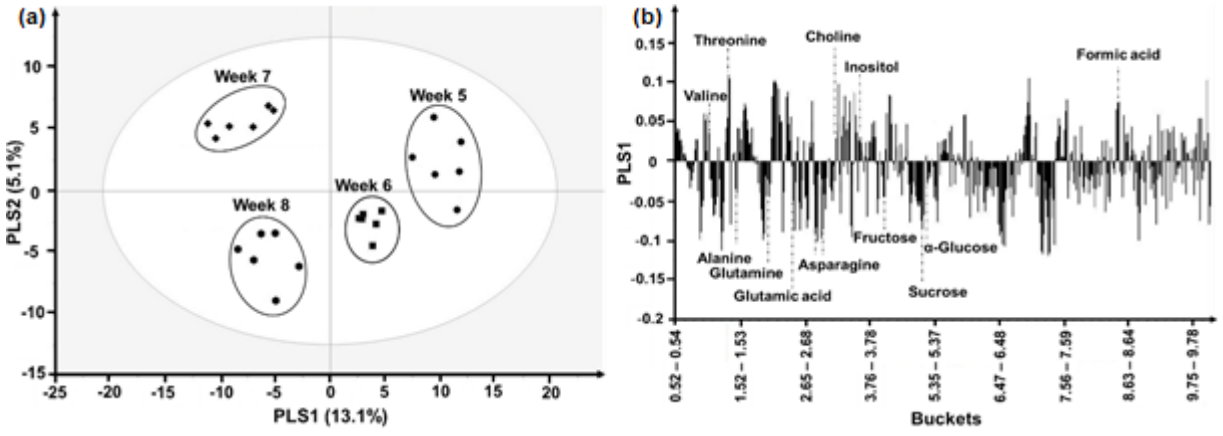

Fig 3. PLSDA score (a) and loading (b) plots from ^{1}H-NMR spectra of water extracts of *bedrobinol* trichomes

metabolites, were found contributing to the discrimination of the metabolic trichomes. Beside those, formic acid, an unquantified metabolite, was also detected giving a contribution in the loading plot. These results indicated that concentrations of these compounds were diverse over the flowering weeks and confirmed the results of the quantitative analysis as explained before.

The results of multivariate data analysis revealed that the trichome metabolomes of *C. sativa* var. *bedrobinol*, which is the THC-type, were varied during the last 4 weeks of the flowering period. These results were similar to the trichome metabolomes of *C. sativa* var. *bediol* which is the intermediate-type [19]. Thus, it indicated that during the flowering period, the metabolite production either in the trichomes of the THC-type *Cannabis* or the intermediate-type depends on the time.

C. sativa possesses 2 growth periods, vegetative and generative or flowering. The plant grows rapidly in the first period and then synthesizes cannabinoids in a large amount in the flowering period. The previous report showed that cannabinoid production in the flower of *C. sativa* var. *bediol* and *bedrobinol* grew under the standardized condition and genetic homogeneity, increased with the time of flowering and achieved the highest level over the last 4 flowering weeks [17]. The responsible tissue in the *Cannabis* flower for producing cannabinoids is the trichome [26]. The pattern of cannabinoid biosynthesis in the trichomes of *C. sativa* var. *bediol* over the second period had been analyzed previously using the ^{1}H-NMR-based metabolomics combined with the real-time PCR analysis [19]. Meanwhile, the pattern of cannabinoid production in the trichomes of THC-type *Cannabis*, var. *bedrobinol*, during the flowering stage was explained in this work using ^{1}H-NMR based metabolomics approach. Therefore, this report may lead to a better understanding of the cannabinome.

■ CONCLUSION

In this report, metabolite profiles of the trichomes of *C. sativa* var. *bedrobinol* over the last 4 weeks of the flowering season were successfully monitored by ^{1}H NMR-based metabolomics. The trichomes of *C. sativa* var. *bedrobinol* during the flowering period biosynthesized metabolites in different amounts depending on the time. Moreover, THCA was found as the most crucial discriminant compound in the classification. This report sheds more light on metabolite profiles of the trichomes of THC-type *Cannabis* during the flowering period.

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