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Profiling and Quantification of Phytocannabinoids for the Classification of *Cannabis sativa* L. Varieties

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Abstract: Whether cannabis is one species or more, is a long-standing topic of debate within the research community. However, according to the current scientific consensus, cannabis is considered as a single but highly variable species, *Cannabis sativa* L. On the other hand, cannabis can also be classified based on its cannabinoid profile, which is more relevant when the goal is to develop cannabis-based phytopharmaceuticals. Once chemically analyzed, screened, and selected, high-yielding mother plants can be mass-propagated through micropropagation or vegetative propagation to ensure batch-to-batch consistency, as well as to maintain quality and efficacy. In this study, plants of different cannabis varieties/chemovars were grown from seeds. Once identified, male plants were removed from the growing area, while only female plants were retained for future research. For the initial screening, biomass samples collected from female plants were analyzed for the cannabinoid content using GC-FID, and the elite mother plants from each variety were further selected. Mother plants were mass-propagated through micropropagation and grown outdoors until they reached maturity. The harvested biomass samples of these plants were then analyzed using GC-FID and NMR spectroscopy. Based on the results, plants were classified into three distinct chemotypes: high THC, intermediate, and high CBD, with clear differentiation. The living germplasm of these plants are further conserved in-vitro for future use.

Keywords: *Cannabis sativa* L., Cannabidiol, Δ^9- Tetrahydrocannabinol, GC-FID analysis, NMR-spectroscopy, Principal Component Analysis (PCA)

INTRODUCTION

Cannabis is one of the oldest medicinal plants known to man. The plant found its origin in Asia and its use spread to the Middle East and later to Europe and the USA. The plant has been recommended by many traditional healers and herbalists for the treatment of a variety of ailments such as headaches, asthma, diarrhea, constipation, pain, and anxiety – to mention just a few (Russo 2017, Zuardi 2006).

The first study to approach defining the structure of the active compounds in cannabis took place in the 1940s, when cannabidiol (CBD) was first isolated by Adams and his team (Adams et al., 1940). However, it was Mechoulam and Shvo who fully elucidated its chemical structure in 1963. The structure of THC was determined by Gaoni and Mechoulam in 1964 (Figure 1). Scientific investigations with cannabis and the cannabinoids have exploded since then. A total of 595 constituents have been isolated from *Cannabis sativa* so far, out of which 129 are Phytocannabinoids (ElSohly 2023).

Cannabis is a highly variable plant species that belongs to the family Cannabaceae. The

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question of whether the genus Cannabis includes one or multiple species has been a subject of long-standing debate and still remains a point of contention (Watts 2006, Piomelli and Russo 2016). The Swedish botanist Carl Linnaeus in 1737 classified cannabis as a single species, while Lamarck, in 1785, distinguished between the 'Indian cannabis strain' and 'European hemp,' giving the former the specific name *Cannabis indica*. In 1976, Small and Cronquist proposed cannabis as a single species (monospecific concept) and categorized it into two subspecies: *Cannabis sativa* L. subsp. *indica* (Lam.) and *C. sativa* L. subsp. *sativa*.

Hillig (2005), based on allozyme data, demonstrated that cannabis originated from two major gene pools and classified *C. sativa* and *C. indica* as separate species. Recent studies by Hillig and Mahlberg (2004), Hillig (2005), McPartland and Guy (2004), and Clarke and Merlin (2016) support a two- or three-species interpretation of cannabis. In contrast, Small (1976, 2015) considers cannabis to be a single, highly variable species, *Cannabis sativa* L. The taxonomic debate continues regarding how to assign scientific names to different cannabis strains, particularly those with varying morphological and chemical profiles, such as the modern hybrid varieties. Nevertheless, based on current scientific consensus from studies of morphology, anatomy, phytochemistry, and genetics, cannabis is generally regarded as a single, highly polymorphic species, *C. sativa* L. This species includes varieties such as *C. sativa* var. *sativa*, *C. sativa* var. *indica*, and *C. sativa* var. *ruderalis*. The botanical nomenclature of cannabis is described in Table 1. This plant is also known by many local common names, some of which are listed in Table 2.

On the other hand, cannabis can also be classified based on its cannabinoid profile. In general, cannabis is categorized into three different popular chemotypes *i.e.* high THC varieties, high CBD varieties, and mix or intermediate varieties (Figure 2). However, cannabis can also be selectively bred for chemotypes high in other cannabinoids such as Tetrahydrocannabivarin (THCV), Cannabichromene (CBC), Cannabigerol (CBG), and Cannabinol (CBN).

In this study, plants of different cannabis varieties are grown from seeds. Based on cannabinoids profile elite mother plants of each variety were selected and multiplied by in-vitro propagation technique. Fully developed plants were transplanted outdoors and grown till their maturity. Samples collected from mature female plants were analyzed (by GC/FID and NMR-spectroscopy) and categorized into three distinct groups (high THC, intermediate, and high CBD) based on their chemical profiles for future use.

MATERIAL AND METHODS

Plants from thirteen different varieties (Mx, B4, B5, V3-15, V3-22, V6-8, V6-13, A-17, A-18, V1-16, V1-19, V1-20 and V1-30) were grown from seeds in 4" jiffy pots in a climate-controlled growing room. Healthy, well-rooted plants were then transplanted into 14" grow-pots for further propagation under a vegetative light (18-hour photoperiod) environment. After reaching the desired vegetative growth, the plants were exposed to a flowering light cycle (12-hour photoperiod). Once flowering occurred, male plants were removed from the growing area. Only female plants from each variety were selected for further propagation. Among these female plants, a high-yielding elite mother plant was identified for each variety based on their cannabinoid content using GC/FID analysis. Vegetative tissues from these mother plants were then used for micropropagation of the respective varieties.

Tissue Culture of Elite Mother Plants from Different Varieties

The apical nodal segments from selected plants of each variety were utilized for micropropagation following Lata et al. (2009). The in-vitro rooted plants were acclimatized indoors until they achieved the desired vegetative growth, after which they were transplanted outdoors in the field. These plants were then grown until they reached full maturity.

GC-FID Analysis of Tissue Culture Raised Plants

Biomass samples of flower buds from plants raised through micropropagation from all cannabis varieties were collected, dried overnight at 125 ± 5°F, and then used for cannabinoid analysis.

Sample Preparation for Analysis

Samples were manicured by sieving (14 mesh) to remove the stems and seeds (if any), and duplicate 100 mg samples from each exhibit were weighed for analysis. Each of the two 100 mg samples was extracted with 3 mL of the internal standard solution [4-androstene-3,17-dione (IS), at 1 mg/mL in $CHCl_3$/MeOH (1:9)] at room temperature for 1 h. The extract was then filtered and the filtrate was analyzed by gas chromatography with flame ionization detection (GC/FID), using our previously reported and validated method (Mehmedic et al. 2010, ElSohly et al. 2000, 2016, Geweda et al. 2024).

Instrumentation and Conditions

All samples were analyzed using a Varian 3380 gas chromatograph equipped with a Varian CP-8400 automatic liquid sampler, dual capillary injectors, and dual flame ionization detectors (GC/FID). The column was a 15 m × 0.25 mm DB-1, 0.25 µ film. Data were recorded with a Dell Optiplex GX1 computer with Microsoft Windows 98 and Varian Star (version 5.31) workstation software. Technical-grade helium was used as the carrier gas. A high-capacity oxygen trap was located in the helium line. Helium was used as the detector make-up gas. Hydrogen and compressed air were used as the combustion gases. The method was used to quantitate six major cannabinoids; namely, Δ^9 -tetrahydrocannabinol (Δ^9-THC), cannabidiol (CBD), cannabinol (CBN), cannabichromene (CBC), cannabigerol (CBG), and tetrahydrocannabivarin (THCV). Direct injection of cannabis extract into the GC results in decarboxylation of the cannabinoid acids, therefore, the concentration measured is for the total cannabinoids (free and acids). Quantitative values are based on peak area ratios relative to the area of the internal standard peak (4-androstene-3,17-dione) contained in the extraction solvent.

Calculation of Cannabinoid Concentration

Quantitative values (% w/w) are computer-generated based on the analyte/internal standard area ratio, with each cannabinoid having a response factor of 1.0. The concentration of a specific cannabinoid is calculated as follows:

$$\text{Cannabinoid (\%)} = \{\text{Peak area (cannabinoid)} / \text{Peak area (ISTD)}\} \times \{\text{Amount (ISTD)} / \text{Amount (sample)}\} \times 100$$

The method has been validated to meet FDA-GMP requirements, ensuring its reliability and consistency for pharmaceutical use.

NMR Spectroscopy Analysis of Tissue Culture Raised Plants

The fresh biomass samples were freeze dried, then extracted with methanol. The methanol extracts of the 41 cannabis biomass samples were analyzed by using *Nuclear Magnetic Resonance (NMR)* spectroscopy following Zhao et al. 2022. The ^1H NMR spectra were processed using Mnova software, and the data set was imported for multivariate statistical analysis which was conducted by using the software SIMCA-P+ (v. 13.0, Umetrics). The data is used to classified cannabis samples to different Chemotype groups based on their cannabinoid profile.

Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was performed on the data obtained by NMR analysis to identify clusters of samples with similar chemical compositions to further confirmation of the GC-FID results.

RESULTS AND DISCUSSION

Indeed, botanical classification plays a crucial role in understanding diversity and has its value in the plant kingdom. Plants that exhibit dioecious, and both dioecious and monoecious characteristics, present significant challenges due to their variable reproductive traits, environmental influences, genetic factors, and potential for hybridization. To address these challenges, modern taxonomic practices increasingly incorporate genetic analysis, ecological observations, and phylogenetic studies to better understand the complex relationships between these plants and to offer more comprehensive classifications. In the case of cannabis, it becomes even more challenging due to its wide distribution across the globe in diverse habitats ranging from the foothills of Himalayan alpines to the tropics.

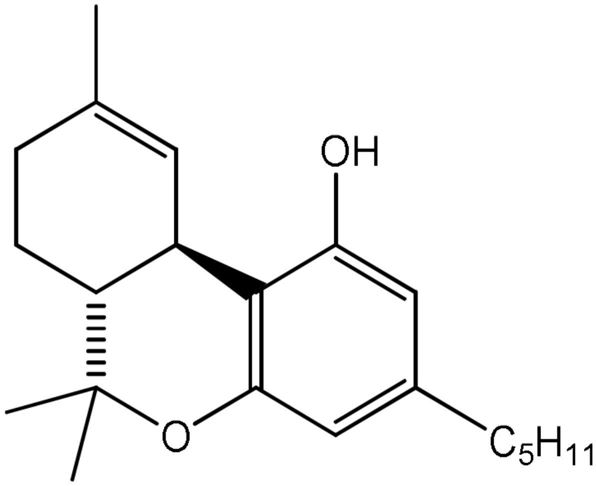
Cannabis is classified as an annual plant, by completing its entire life cycle from germination to the production of seeds within a single growing season. After seed production, the plant normally dies in nature (if not harvested), and new plants grow from the seeds in the next season. It is a dioecious plant having individual plants being either male or female. Male plants produce pollen, while female plants produce seeds after fertilization. However, in some cases, Cannabis exhibits monoecious characteristics, where a single plant may contain both male and female reproductive organs. These plants are often referred to as "hermaphrodites," and this phenomenon can occur due to environmental stressors (e.g., inadequate lighting or nutrient imbalance), genetic factors, or cultivation practices. While this can lead to seed production on a single plant, it is typically considered undesirable in cannabis cultivation for seedless female plants (sinsemilla), which are sought after for higher concentrations of cannabinoids.

Furthermore, cannabis is characterized highly allogamous by nature, meaning it predominantly relies on cross-pollination for fertilization, enhancing genetic diversity within the population. Therefore, it is very difficult to maintain the chemical profile of any cannabis variety/cultivar, if different varieties (raised through seeds) are being grown side by side. More than 700 varieties/cultivars of cannabis are already documented by 2014 and these numbers are increasing every day (Erkelens and Hazekamp, 2014).

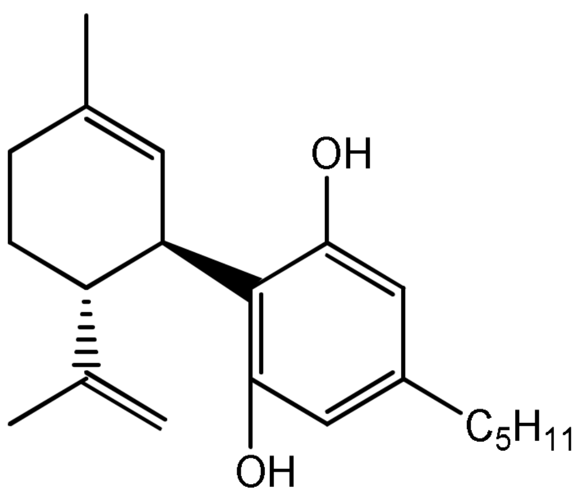
In view of complex botanical characterization, a metabolomics approach is suggested by many authors for the classification of cannabis varieties or cultivars (Chirambo et al. 2024, Hazekamp et al. (2016), Piomelli and Russo (2016). In this study, we used 13 different cannabis cultivars for the classification based on their chemical profiles. Plants were raised from seeds. Male plants were removed and high cannabinoids yielding female mother plants were selected based of GC/FID analysis. Selected mother plants were mass-propagated using micropropagation. Fully rooted healthy plants were cultivated outdoor in field till maturity. The biomass samples from well identified randomly selected healthy plants from each variety were collected at vegetative and flowering stages. These samples were analyzed for the cannabinoid analysis using GC/FID.

Based on the diversity in cannabinoids, especially in THC and CBD content, samples were successfully classified into three different distinct groups *i.e.* High THC and low CBD variety, Intermediate variety (THC~CBD), and high CBD and low THC varieties (Figure 2). Cultivar Mx exhibited highest THC content with 13.12% and 0.02% CBD in clone ID Mx-P1. This cultivar/chemovar exhibited a unique cannabinoid profile and was kept in a distinct (High THC) category (Figure 3A). Six cultivars B4, B5, V3-15, V3-22, V6-8, V6-13, A-17 and A-18 were identified as intermediate varieties/chemovars with THC and CBD content highest in clone ID B4-P2 (7.56% THC and 7.51% CBD) and lowest in V6-13-P2 (2.83% THC and 2.86% CBD). All the chemovars in this group maintained similar ratio of THC and CBD at vegetative and flowering stages of growth (Figure 3B). Cultivars V1-16, V1-19, V1-20 and V1-30 were characterized as high CBD chemovars with highest CBD content in Clone ID V1-30-P2 (7.83% CBD and 0.25% THC, Figure 3C).

A portion of the fresh biomass samples used for GC-FID analysis was also used for NMR spectroscopy. After harvesting, the samples were quickly freeze-dried and then extracted with methanol for further analysis by NMR. Tetrahydrocannabinolic acid (THCA), Cannabidiolic acid (CBDA), or a combination of both THCA and CBDA were found to be the primary components in the extracts, while the levels of neutral cannabinoids were very low, indicating that decarboxylation occurred minimally during sample preparation. Principal Component Analysis (PCA) was performed on the NMR data to identify sample clusters with similar chemical profiles, further supporting the GC-FID results. The PCA scatter plots revealed three distinct chemotype groups: (i) high THCA chemotypes, (ii) intermediate chemotypes with



Δ^9-Tetrahydrocannabinol
(Δ^9-THC)



Cannabidiol
(CBD)

Figure 1: Chemical structures of two major cannabinoids in *Cannabis sativa* **L.**

both THCA and CBDA, and (iii) high CBDA chemotypes (Figure 4).

These results confirm a clear-cut quantitative and qualitative differences in cannabinoid profile and content in different cannabis varieties/ cultivars or chemovars. Based on the results mother (female) plants were identified and conserved in- vitro for future use without any confusion.

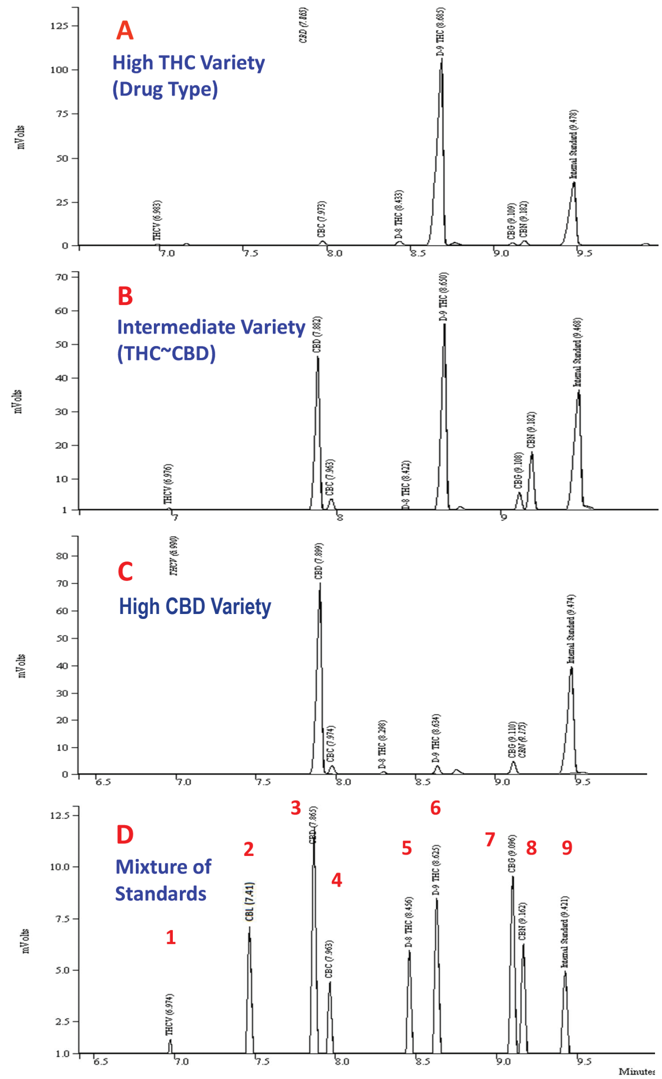


Figure 2: Representative gas chromatography flame ionization detector (GC-FID) analysis of cannabis biomass. (A) high THC variety, (B) intermediate (THC~ CBD) variety, (C) high CBD variety and (D) GC-FID profile of a mixture of standard cannabinoids 1: THCV, 2: CBL, 3: CBD, 4: CBC, 5: Δ^8-THC, 6: Δ^9-THC, 7: CBG, 8: CBN and 9: internal standard (IS)

Table 1. Botanical nomenclature

Category	*Botanical Nomenclature*
Kingdom	Plantae - Plants
Subkingdom	Vascular plants
Superdivision	Seed plants
Division	Flowering plants
Class	Dicotyledons
Subclass	Hamamelididae
Order	Urticales
Family	Cannabaceae
Genus	Cannabis
Species	*Cannabis sativa* L.

Table 2: Popular names

Language	*Name*
Arabic	Bhang, Hashish, Kenneb, Tîl
Chinese	Xian ma
Danish	Hemp
Dutch	Hennep
English	Hemp, Marihuana
French	Chanvre, Chanvre d'Inde¸ Chanvre
German	Hanf, Haschisch, Indischer hanf
Hindi	Bhang, Charas, Ganja
Portuguese	Cânhamo, Maconha
Spanish	Cáñamo, Grifa, Hachís, Marijuana

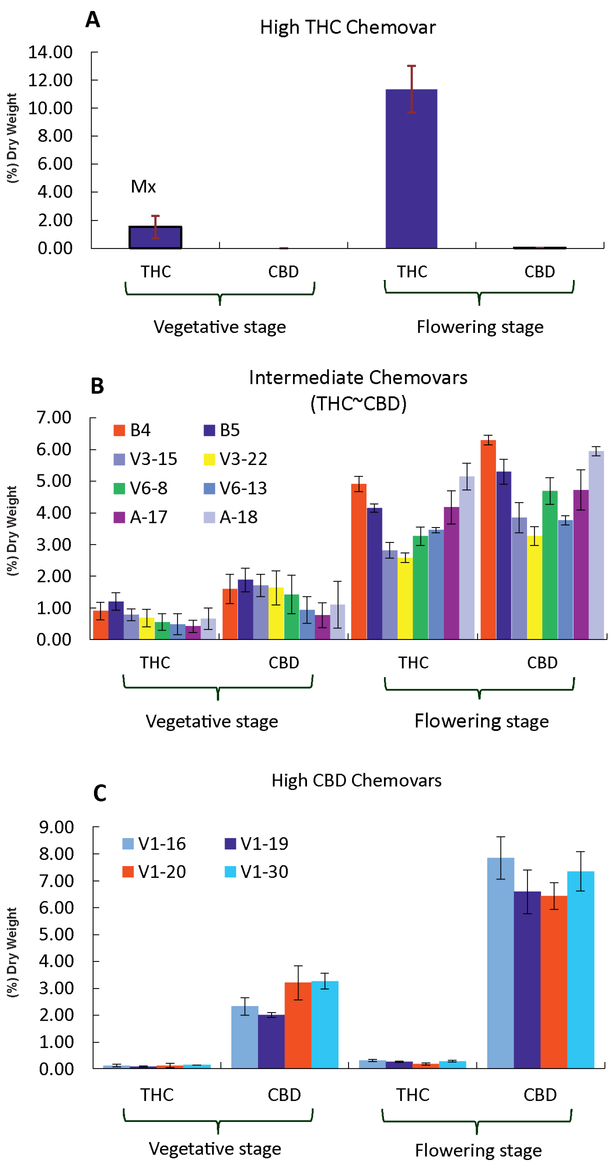


Figure 3: Δ⁹-THC and CBD content in different varieties of *C. sativa* at vegetative and flowering stages. Plants were classified in three distinct chemotypes/chemovars based on their THC and CBD content using GC-FID analysis. (A) high THC and low CBD chemotype, (B) intermediate chemotypes (THC~CBD) and (C) high CBD and low THC chemotypes

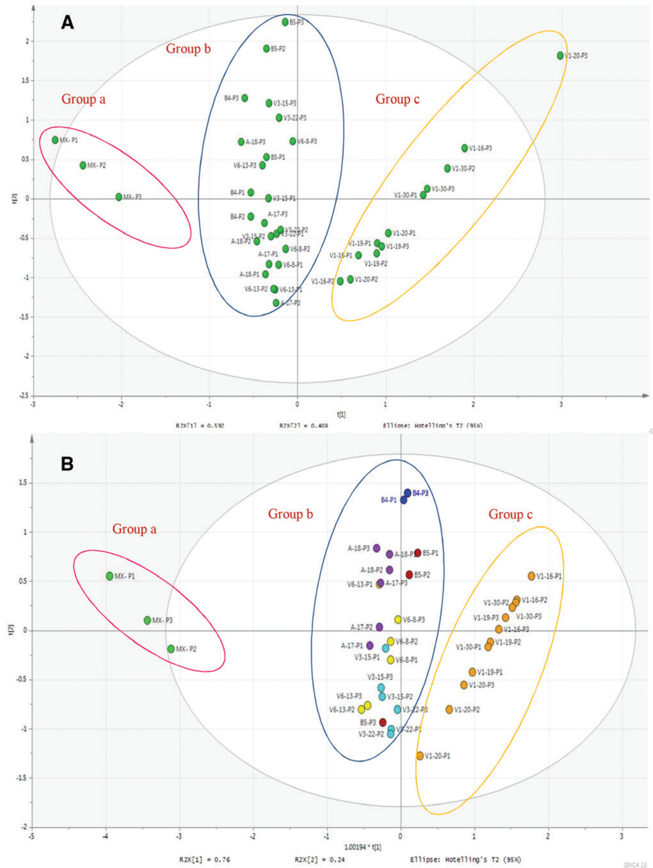


Figure 4: Principal Component Analysis (PCA) scores scatter plots for different varieties of *Cannabis sativa* L. based on their cannabinoid (acidic) profiles using NMR data at (A) vegetative and (B) flowering stages. Group a: high THC chemovar, b: intermediate chemovars (THC~CBD), and c: high CBD chemovars

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