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Development and Validation of a GC-FID Method for the Quantitation of 20 Different Acidic and Neutral Cannabinoids

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ABSTRACT

For decades, *Cannabis sativa* had been illegal to sell or consume around the world, including in the United States. However, in light of the recent 2018 Farm Bill and the legalization of hemp across the US, various cannabis preparations have flooded the market, making it essential to be able to quantitate the levels of the different acidic and neutral cannabinoids in *C. sativa* and to have a complete cannabinoid profile of the different chemovars of the cannabis plant. A GC-FID method was developed and validated for the analysis of 20 acidic and neutral cannabinoids as trimethylsilyl (TMS) derivatives. The analyzed cannabinoids include cannabidivarinic acid (CBDVA), cannabidiolic acid (CBDA), cannabinolic acid (CBNA), cannabielsoic acid (CBEA), cannabicyclolic acid (CBLA), cannabichromenic acid (CBCA), *trans*-Δ^9-tetrahydrocannabivarinic acid (Δ^9-THCVA), *trans*-Δ^9-tetrahydrocannabinolic acid A (Δ^9-THCAA), cannabigerolic acid (CBGA), cannabidiol (CBD), cannabicyclol (CBL), cannabidivarin (CBDV), *trans*-Δ^9-tetrahydrocannabivarin (THCV), cannabichromene (CBC), *trans*-Δ^8-tetrahydrocannabinol (Δ^8-THC), *trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabigerol (CBG), cannabinol (CBN), cannabicitran (CBT), and cannabielsoin (CBE). The method limit of detection (LOD) was as low as 0.1 µg/mL, while the limit of quantitation ranged from 0.25 µg/mL to 0.5 µg/mL. The precision (%RSD) was < 10%, while trueness ranged from 90–107%. The developed method is simple, accurate, and sensitive for the quantitation of all 20 acidic and neutral cannabinoids. Finally, the proposed method was successfully applied to the quantitation of the cannabinoids in different cannabis chemovars grown at the University of Mississippi.

Introduction

Cannabis (*Cannabis sativa* L., Cannabaceae) has been legalized in most states of the US, either for medical or recreational uses [1]. It is indicated to be used for the treatment of an enormous array of health problems including pain, inflammation, amenorrhea, and arthritis [2]. The extended history of medical use of cannabis in the treatment of many symptoms and diseases is attributed to its rich content of phytochemicals, namely, cannabinoids in addi-

ABBREVIATIONS

BSTFA	N,O-Bis (trimethylsilyl)-trifluoroacetamide
C. sativa	*Cannabis sativa*
CBC	cannabichromene
CBCA	cannabichromenic acid
CBD	cannabidiol
CBDA	cannabidiolic acid
CBDV	cannabidivarian
CBDVA	cannabidivarinic acid
CBE	cannabielsoin
CBEA	cannabielsoic acid
CBG	cannabigerol
CBGA	cannabigerolic acid
CBL	cannabicyclol
CBLA	cannabicyclolic acid
CBN	cannabinol
CBNA	cannabinolic acid
CBT	cannabicitran
DMAP	dimethylaminopyridine
THCV	*trans*-Δ^9-tetrahydrocannabivarian
TMS	trimethylsilyl
Δ^8-THC	*trans*-Δ^8-tetrahydrocannabinol
Δ^9-THC	*trans*-Δ^9-tetrahydrocannabinol
Δ^9-THCAA	*trans*-Δ^9-tetrahydrocannabinolic acid A
Δ^9-THCVA	*trans*-Δ^9-tetrahydrocannabivarinic acid

tion to the non-cannabinoids, such as terpenes and flavonoids, and to a lesser extent alkaloids [3].

The most important constituents of cannabis are the cannabinoids, such as Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive cannabinoid currently approved to treat adverse effects associated with chemotherapy, in addition to the non-psychoactive component, cannabidiol (CBD), which is approved by FDA for the treatment of seizures [4, 5].

Cannabis has been classified into three chemovars. The first class is the high-THC chemovar that contains Δ^9-THC as the predominant cannabinoid. The second chemovar is the CBD dominant, commonly known as hemp, with < 0.3 % THC level. The third chemovar is an intermediate chemovar with balanced levels of both THC and CBD with levels ≥ 1 % of each [6].

Currently, hemp is extensively used in the manufacture of a variety of commercial products containing CBD. These include edibles, topicals, cosmetics, and hair products, as well as a variety of foods and drinks [7–9]. The US Food and Drug Administration (FDA) approved some cannabis-based medicines like dronabinol (Marinol) and nabilone (Cesamet) for the treatment of chemotherapy-induced nausea and vomiting in acquired immune deficiency syndrome (AIDS) patients [10, 11]. Also, Epidiolex® (CBD-solution) has been approved by the FDA for the treatment of Lennox-Gastaut and Dravet's syndrome, especially in children [12, 13]. According to the European Medicines Agency (EMA), there is only one cannabis-based medicine – Epidiolex® – which can be used in the European Union for the treatment of rare types of epilepsy (Lennox-Gastaut and Dravet's syndrome) and could also be used in the treatment of tuberous sclerosis [14].

Other cannabinoids have been shown to have important biological activities. For example, cannabigerol (CBG) has been tested in combination with THC and CBD as an anti-cancer agent and found to be a potent therapeutic agent in the treatment of glioblastoma [15]. Cannabidivarin (CBDV) was found to be an effective therapeutic agent in the treatment of Rett syndrome [16]. Also, Δ^9-tetrahydrocannabivarin (THCV) was clinically reported to manage obesity and diabetes [17]. Cannabichromene (CBC) might be used as an alternative therapy in the treatment of acute respiratory distress syndrome (ARDS) [18]. In addition, CBC was reported as a cannabinoid CB2 receptor agonist and hence plays an important role in the modulation of inflammation [19].

Therefore, the chemical profile of cannabis, as a botanical drug, needs to be delineated and the reproducibility of the composition from batch to batch needs to be established by chemical analysis.

Searching the literature, many analytical methods were used for the chemical profiling of the cannabinoid content in cannabis. Some of these methods don't require derivatization before analysis, such as HPLC-UV and LC-MS, but might require more prepurification steps [20–28]. LC-MS/MS methods were used for the determination of illicit drugs in biological matrices [29]. In addition, LC-MS/MS was used for the analysis of most common narcotic substances in seized illicit materials and phytocannabinoids in oil-based preparations [30, 31].

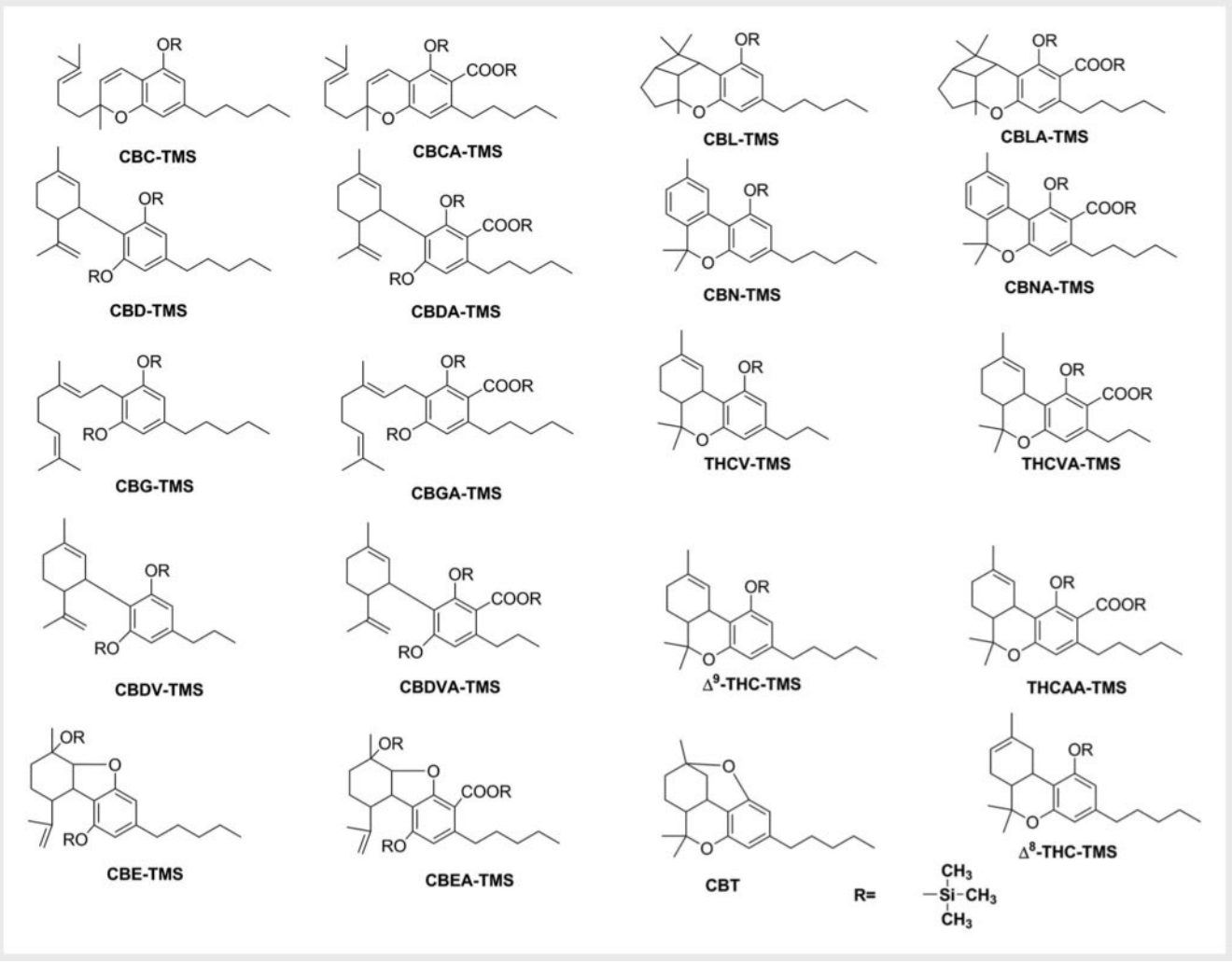
While HPLC is recognized and used by many investigators as the primary method for analysis of cannabinoids because of its simplicity and ease of use, including no need for derivatization, the GC/FID outlined here offer a valid alternative with the advantage of increased sensitivity, peak resolution, and wide dynamic range [32].

GC-FID and GC-MS have been frequently used for the determination of cannabinoids [33, 34]. However, in general, GC techniques are unable to determine acids without prior derivatization, as decarboxylation occurs at the high temperature of the GC inlet [35–38].

To avoid decarboxylation and/or degradation of cannabinoids, chemical derivatization (silylation as TMS derivatives) is used. To the best of our knowledge, cannabinoids are most effectively identified and quantified using silylated derivatives [39, 40]. Derivatization enhances chromatography and repeatability by capturing polar or reactive groups in the target analyte. Furthermore, derivatization makes the analytes more volatile, therefore easier to be chromatographed at lower temperatures [36, 39, 41, 42].

To determine the intact acidic cannabinoids and avoid the decarboxylation process, samples have to be derivatized, most commonly by the silylation technique using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) [36, 43, 44].

We have previously published a GC-FID method for the analysis of 13 cannabinoids [45]. This study aimed to add another 7 cannabinoids to the previously published method, which enables the analysis of 9 acidic and 11 neutral cannabinoids, namely, CBDVA, THCVA, CBDA, CBCA, Δ^9-THCAA, CBGA, CBNA, CBLA, CBEA, CBDV, THCV, CBD, CBC, Δ^8-THC, Δ^9-THC CBG, CBN, CBL, CBE,



▶ **Fig. 1** Chemical structures of the 20 silylated acidic and neutral cannabinoids.

and CBT, and was applied for the analysis of plant material extracts.

Results and Discussion

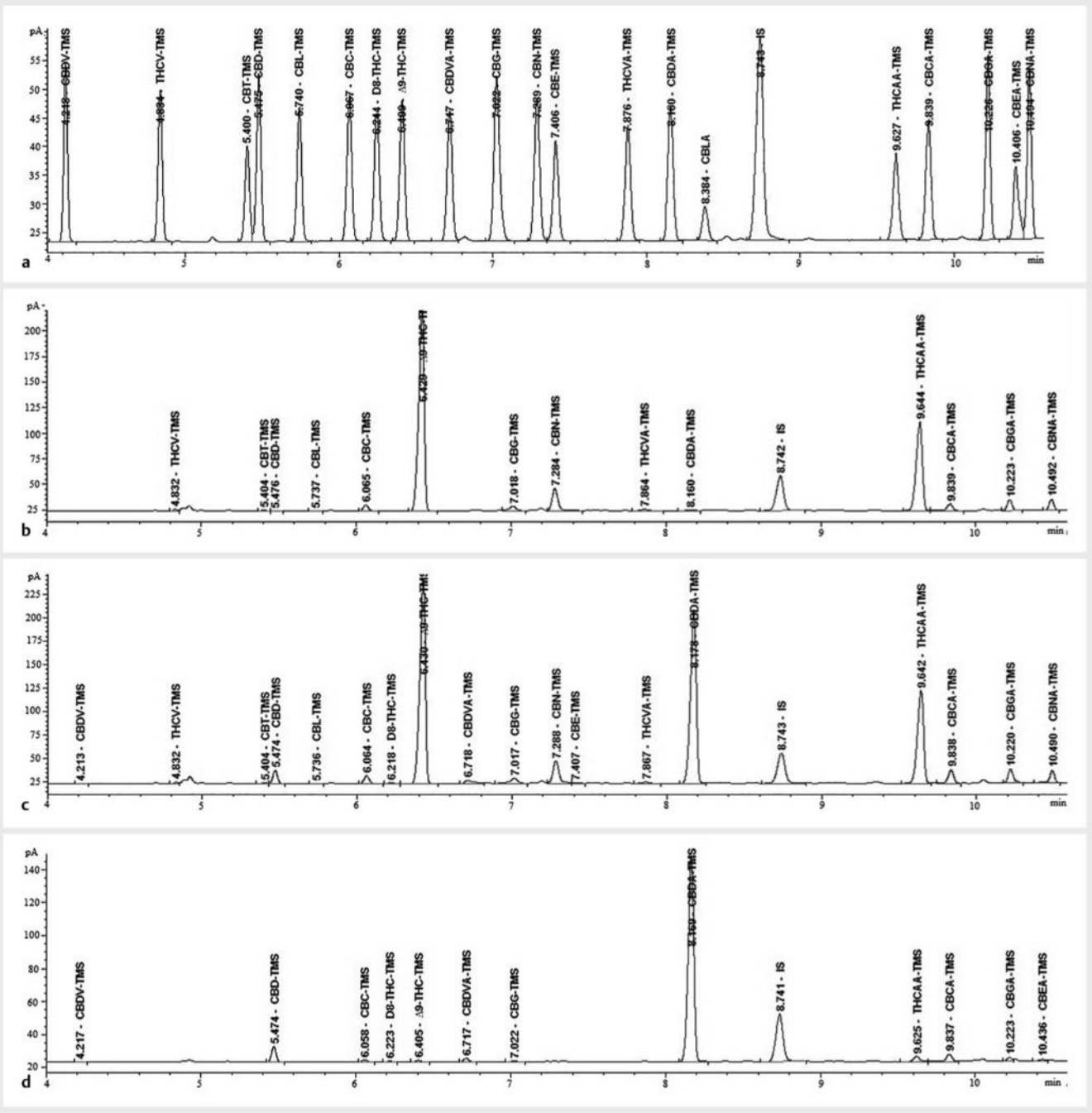
As a continuation of our previous work, we thought it worthwhile to analyze more cannabinoids aiming at getting a better cannabinoid profile using GC-FID [45].

During the growth process of the *C. sativa* plant, phytocannabinoid acids are biosynthesized and, post-harvest, these acids decarboxylate to their neutral analogs when exposed to heat and light. In order to quantify acidic cannabinoids in addition to neutral ones, BSTFA was used as the silylating agent. The high derivatization/silylation rate of BSTFA for both neutral and acidic cannabinoids by reacting with both hydroxyl and carboxylic groups prevents acidic cannabinoids from decarboxylation at the high temperature of the injection port. In addition, silylation increases the method sensitivity and enhances peak symmetry. Dimethyl aminopyridine (DMAP) was used as a base or catalyst during the derivatization process. The chemical structures of the silylated cannabinoids are shown in ▶ **Fig. 1**.

The method was proved to be sensitive, accurate, and fast as the 20 cannabinoid TMS derivatives are eluted in less than 11 min (with a total run of 17.5 min) without sacrificing resolution between adjacent peaks. A GC chromatogram of a mixture of the 20 cannabinoid TMS derivatives is shown in ▶ **Fig. 2a**.

To determine the degree of carry-over, one blank TMS was injected immediately after each sample (**Fig. 1S**, Supporting Information). The blank TMS sample must not show the analytes and/or internal standard peaks at a signal-to-noise (S/N) ratio of ≥ 3. **Fig. 2S** (Supporting Information) shows the peak of IS at the expected retention time. The linear equation regression parameters are shown in ▶ **Table 1**.

In order to confirm the applicability of this work, the developed method was then validated following ICH guidelines [46]. The six-point calibration curves were constructed, in triplicate, for each silylated cannabinoid using the internal standard (IS) method. Calibration curves were linear over the dynamic concentration range of 0.25–25 µg/mL for all silylated cannabinoids except for



▶ **Fig. 2** **a** Representative chromatogram of the standard silylated cannabinoid mixture (25 µg/mL) and IS (50 µg/mL) with respective retention times, **b** representative chromatogram of the high THC chemovar, **c** representative chromatogram of the intermediate chemovar, and **d** representative chromatogram of the high CBD chemovar.

CBE, CBEA, CBN, CBNA, and CBLA, where the dynamic range was 0.5–25 µg/mL. It is obvious that all compounds had a coefficient of determination ($R^2 > 0.999$) indicating an excellent fit of the silylated cannabinoids to the model within the range studied (▶ **Table 1**).

The limits of detection (LOD) and quantitation (LOQ) are shown in ▶ **Table 2**. The overall LOD and LOQ of the method were found to be 0.1 µg/mL and 0.25 µg/mL, respectively, for all cannabinoids except the minors (CBN, CBLA, CBE, CBEA, and CBLA), where the LOQ was 0.5 µg/mL (▶ **Table 2**).

The intra-day trueness (evaluated as % recovery) and precision expressed as relative standard deviation (%RSD) ranged between 90–107% and 0.4–9.10%, respectively, for all cannabinoids. The inter-day trueness and precision ranged from 93–105% and 0.4–5.8%, respectively (▶ **Tables 3** and **4**). These values indicate that

▶ **Table 1** Regression equation parameters for all the tested cannabinoids.

Parameters	Calibration range (µg/mL)	Regression equation (Y): SLOPE (S)	Correlation coefficient (R^2)
CBDV	0.25–25	y = 0.0313 x – 0.0008	1.0000
THCV	0.25–25	y = 0.0270 x – 0.0002	1.0000
CBT	0.25–25	y = 0.0189 x + 0.0005	0.9999
CBD	0.25–25	y = 0.0317 x + 0.0002	0.9999
CBL	0.25–25	y = 0.0252 x + 0.0002	0.9999
CBC	0.25–25	y = 0.0305 x + 0.0022	0.9998
Δ^8-THC	0.25–25	y = 0.0292 x + 0.0028	0.9997
Δ^9-THC	0.25–25	y = 0.0277 x + 0.0031	0.9999
CBDVA	0.25–25	y = 0.0280 x – 0.0005	0.9998
CBN	0.5–25	y = 0.0277 x + 0.0006	0.9999
CBG	0.25–25	y = 0.0318 x – 0.0006	0.9999
CBE	0.5–25	y = 0.023 x + 0.0072	0.9995
THCVA	0.25–25	y = 0.0244 x – 0.0005	0.9999
CBDA	0.25–25	y = 0.0488 x – 0.0014	0.9993
CBLA	0.5–25	y = 0.007 x + 0.0013	0.9997
THCAA	0.25–10	y = 0.0187 x – 0.0015	0.9997
CBCA	0.25–25	y = 0.0212 x + 0.0000	0.9999
CBGA	0.25–25	y = 0.0268 x + 0.0008	0.9998
CBEA	0.5–0.25	y = 0.0126 x – 0.0004	0.9996
CBNA	0.5–25	y = 0.0250 x – 0.0008	1.000

this method is accurate, precise, and acceptable for the quantification of these cannabinoids.

The validated method was subsequently applied for the analysis of plant material from different cannabis chemovars (high-CBD chemovar, intermediate chemovar, and high-THC chemovar). The analysis results are shown in ▶ **Table 5**. The results of the analysis of the three cannabis chemovars in terms of the content of the major cannabinoids (THC and/or CBD) were different. From the results, it is clear that the validated method was able to determine acidic cannabinoids in addition to neutral cannabinoids. The values of THCAA and Δ^9-THC were higher in high-THC chemovar and found to be about 20% and 2%, respectively (▶ **Table 5**, and ▶ **Fig. 2b**). In high-THC chemovar, other cannabinoids found were CBG, THCVA, CBEA, and CBGA. The intermediate chemovar contained nearly equal amounts of CBD/CBDA and Δ^9-THC/THCAA (▶ **Fig. 2c**). The high-CBD chemovar contained high amounts of CBD/CBDA in comparison with other cannabinoids (▶ **Fig. 2d**). The cannabinoids' content of the different chemovars is shown in ▶ **Table 5**.

In conclusion, the developed GC-FID method is simple, accurate, and sensitive for the analysis of 20 acidic and neutral cannabinoids with baseline separation of all analytes. It can be routinely used in laboratories for quick and accurate analysis of cannabinoid content.

Material and Methods

Standards and reagents

Seventeen cannabinoids' reference standards were purchased from Cerilliant. Neutral cannabinoids (CBC, CBL, CBD, CBDV, CBG, CBN, THCV, Δ^8-THC, and Δ^9-THC) were at 1 mg/mL in MeOH, while the acidic cannabinoids (CBCA, CBDA, CBDVA, CBGA, CBLA, CBNA, Δ^9-THCAA, and Δ^9-THCVA) were at 1 mg/mL in MeCN. CBE, CBEA, and CBT were isolated from the cannabis plant material, identified, and confirmed by ^{1}H and ^{13}C NMR (Coy Waller complex, National Center for Natural Products Research (NCNPR), School of Pharmacy, The University of Mississippi), which were prepared at 1 mg/mL in MeCN. The purity of all the reference standards was confirmed using GC-FID and GC-MS (purity > 98%). The internal standard 4-androstene-3,17-dione (I. S.) was purchased from Zhuhai Yuancheng Pharmaceutical and Chemical Company. BSTFA and dimethyl aminopyridine (DMAP) were purchased from Sigma-Aldrich with purity > 99% (confirmed by GC-MS).

MeCN and MeOH were of HPLC grade, while chloroform was of analytical grade and obtained from Fisher Scientific.

▶ **Table 2** Retention times, LOD, and LOQ of the tested cannabinoids and the internal standard (IS).

Compound	Retention time	RTT	LOD (µg/mL)	LOQ (µg/mL)
CBDV	4.218	0.482	0.1	0.25
THCV	4.834	0.553	0.1	0.25
CBT	5.400	0.618	0.1	0.25
CBD	5.475	0.626	0.1	0.25
CBL	5.740	0.657	0.1	0.25
CBC	6.067	0.694	0.1	0.25
Δ^8-THC	6.244	0.714	0.1	0.25
Δ^9-THC	6.409	0.733	0.1	0.25
CBDVA	6.747	0.772	0.1	0.25
CBN	7.022	0.803	0.1	0.50
CBG	7.289	0.834	0.1	0.25
CBE	7.406	0.847	0.1	0.50
THCVA	7.876	0.901	0.1	0.25
CBDA	8.160	0.933	0.1	0.25
CBLA	8.384	0.959	0.1	0.50
THCAA	9.627	1.101	0.1	0.25
CBCA	9.839	1.125	0.1	0.25
CBGA	10.226	1.170	0.1	0.25
CBEA	10.406	1.190	0.1	0.50
CBNA	10.494	1.200	0.1	0.5
IS	8.743	1.000	–	–

RTT = Relative Retention Time; LOD = Limit of Detection; LOQ = Limit of Quantitation

Cannabis plant material

Cannabis plants (*in vitro* micropropagated at the University of Mississippi) were grown in an indoor controlled environment (light with a photoperiod of 16 h, 700 µmol/m^2/s, the temperature was set at 25–30 °C, and relative humidity (60 %). The taxonomy of the plants was recognized by Dr. Suman Chandra and a voucher specimen (S1310V1) was kept at Coy–Waller Laboratory, School of Pharmacy, University of Mississippi, USA.

Instrumentation and column

GC-FID analysis was performed on Agilent 6890 Network GC System (Agilent Technologies) fitted with a 7683B-series injector. Separations were carried out on a DB-1MS column (15 m × 0.25 mm, and 0.25 µm film thickness). Helium was used as the carrier gas at a flow rate of 0.8 mL/min and as detector make-up gas. The inlet temperature was set at 275 °C with a split ratio of 20 : 1. The temperature program started at 190 °C and was held for 1 min, then ramped to 230 °C with a rate of 30 °C/min (held for 2 min). Next, the oven temperature was ramped to 250 °C at a rate of 5 °C/min (held for 1 min), and then the temperature increased at a rate of 20 °C/min to 300 °C (held for 2.75 min). At the end of the gradient, the oven cooled back down to 190 °C. The total run time was 17.5 min. Detector temperature was set at 300 °C and the hydrogen, air, and make-up flow rates were 40, 500, and 27 mL/min., respectively. Data were acquired and analyzed by Agilent ChemStation software (rev. B.04.02).

Internal standard and DMAP preparation

The IS (1 mg/mL of 4-androstene-3,17-dione) was prepared in MeOH and chloroform (9 : 1, v : v). A solution of 2 % DMAP was prepared in MeOH.

Standard solutions preparation

From vials containing individual cannabinoids with a concentration of 1.0 mg/mL, 50 µL were mixed. After evaporation to dryness under nitrogen, the cannabinoid mixture was re-dissolved in 1 mL of MeOH to get a stock standard solution of 50 µg/mL. Serial dilutions were made to prepare the calibration curve points.

Calibration curves and control samples

A six-point calibration curve was prepared from stock standard solution (50 µg/mL cannabinoid mixture) at 0.25–25 µg/mL for all cannabinoids except CBN, CBL, CBE, and CBEA, which were prepared from the stock standard solution (50 µg/mL cannabinoid

▶ **Table 3** Intra-day precision and trueness (n = 30).

Compound	Concentration (µg/mL)	Batch 1				Batch 2				Batch 3				Batch 4				Batch 5			
		Mean	SD	Trueness	Precision (%RSD)	Mean	SD	Trueness	Precision (%RSD)	Mean	SD	Trueness	Precision (%RSD)	Mean	SD	Trueness	Precision (%RSD)	Mean	SD	Trueness	Precision (%RSD)
CBDV	1.25	1.24	0.03	101%	2.60%	1.21	0.01	98%	1.00%	1.22	0.02	99%	1.20%	1.2	0.01	97%	1.10%	1.25	0.01	101%	1.10%
	2.5	2.5	0.06	101%	2.50%	2.45	0.05	99%	2.20%	2.4	0.04	97%	1.70%	2.49	0.06	100%	2.40%	2.5	0.05	101%	2.20%
	5	5.11	0.11	101%	2.20%	5.07	0.03	100%	0.60%	4.9	0.03	97%	0.70%	5.21	0.04	103%	0.80%	5.17	0.05	102%	0.90%
THCV	1.25	1.22	0.02	98%	1.20%	1.2	0.01	96%	1.20%	1.19	0.02	95%	1.30%	1.24	0.01	99%	1.10%	1.25	0.01	100%	1.10%
	2.5	2.5	0.05	100%	2.10%	2.46	0.05	98%	2.10%	2.48	0.04	99%	1.80%	2.5	0.06	100%	2.30%	2.51	0.05	101%	2.10%
	5	5.13	0.02	103%	0.50%	5.1	0.03	102%	0.60%	5.2	0.04	104%	0.80%	5.17	0.04	103%	0.80%	5.2	0.05	104%	0.90%
CBT	1.25	1.22	0.02	98%	1.30%	1.19	0.01	95%	1.10%	1.2	0.01	96%	1.20%	1.19	0.01	95%	1.20%	1.26	0.03	101%	2.40%
	2.5	2.5	0.05	100%	1.90%	2.45	0.05	98%	2.00%	2.48	0.04	99%	1.50%	2.48	0.05	99%	2.10%	2.5	0.05	100%	1.90%
	5	5.14	0.03	103%	0.50%	5.09	0.03	102%	0.60%	5.21	0.04	104%	0.80%	5.22	0.04	104%	0.80%	5.17	0.05	103%	0.90%
CBD	1.25	1.21	0.02	97%	1.50%	1.19	0.01	95%	1.30%	1.19	0.02	95%	1.30%	1.16	0.01	93%	1.10%	1.23	0.01	99%	0.60%
	2.5	2.49	0.05	100%	2.00%	2.46	0.05	98%	2.10%	2.48	0.04	99%	1.80%	2.47	0.06	99%	2.40%	2.5	0.05	100%	2.10%
	5	5.14	0.03	103%	0.50%	5.1	0.03	102%	0.60%	5.2	0.04	104%	0.70%	5.22	0.04	104%	0.80%	5.16	0.06	103%	1.20%
CBL	1.25	1.22	0.01	98%	1.20%	1.2	0.01	96%	1.10%	1.21	0.03	97%	2.70%	1.24	0.01	99%	1.00%	1.3	0.12	104%	9.10%
	2.5	2.51	0.05	100%	2.00%	2.46	0.06	98%	2.30%	2.49	0.04	99%	1.70%	2.5	0.06	100%	2.40%	2.52	0.06	101%	2.40%
	5	5.16	0.02	103%	0.50%	5.11	0.03	102%	0.60%	5.22	0.04	104%	0.70%	5.15	0.04	103%	0.90%	5.19	0.05	104%	0.90%
CBC	1.25	1.21	0.01	100%	1.20%	1.19	0.02	98%	1.30%	1.19	0.02	98%	1.50%	1.23	0.01	102%	1.10%	1.18	0.01	97%	1.10%
	2.5	2.49	0.05	100%	2.10%	2.46	0.06	99%	2.30%	2.48	0.04	100%	1.70%	2.5	0.06	100%	2.40%	2.49	0.06	100%	2.30%
	5	5.14	0.03	100%	0.50%	5.11	0.03	100%	0.60%	5.21	0.04	101%	0.70%	5.15	0.04	100%	0.80%	5.29	0.06	103%	1.20%
Δ^8-THC	1.25	1.23	0.03	98%	2.40%	1.24	0.02	99%	1.40%	1.23	0.01	98%	1.20%	1.24	0.01	100%	1.20%	1.18	0.02	94%	1.30%
	2.5	2.5	0.06	100%	2.40%	2.47	0.06	99%	2.30%	2.48	0.04	99%	1.80%	2.5	0.06	100%	2.40%	2.49	0.06	100%	2.50%
	5	5.14	0.02	103%	0.40%	5.05	0.03	101%	0.60%	5.13	0.04	103%	0.70%	5.15	0.05	103%	0.90%	5.26	0.05	105%	0.90%
Δ^9-THC	1.25	1.24	0.02	99%	1.40%	1.22	0.02	97%	1.60%	1.21	0.02	97%	1.30%	1.25	0.02	100%	1.40%	1.26	0.02	101%	1.40%
	2.5	2.51	0.06	100%	2.40%	2.48	0.06	99%	2.30%	2.5	0.05	100%	2.00%	2.52	0.08	101%	3.10%	2.54	0.08	102%	3.00%
	5	5.15	0.03	103%	0.50%	5.14	0.03	103%	0.70%	5.24	0.04	105%	0.80%	5.2	0.05	104%	0.90%	5.22	0.05	104%	1.00%

continued

▶ **Table 3** *Continued*

Compound	Concentration (µg/mL)	Batch 1				Batch 2				Batch 3				Batch 4				Batch 5			
		Mean	SD	Trueness	Precision (%RSD)	Mean	SD	Trueness	Precision (%RSD)	Mean	SD	Trueness	Precision (%RSD)	Mean	SD	Trueness	Precision (%RSD)	Mean	SD	Trueness	Precision (%RSD)
CBDVA	1.25	1.15	0.02	92%	1.60%	1.2	0.02	96%	1.60%	1.13	0.02	91%	1.80%	1.14	0.02	91%	1.70%	1.2	0.02	96%	1.50%
	2.5	**2.41**	**0.07**	**97%**	**3.00%**	**2.41**	**0.07**	**96%**	**2.90%**	**2.39**	**0.05**	**96%**	**2.10%**	2.38	0.07	95%	3.00%	2.45	0.07	98%	2.90%
	5	5.09	0.03	102%	0.60%	4.98	0.03	100%	0.60%	5.18	0.04	104%	0.70%	5.2	0.04	104%	0.80%	5.1	0.05	102%	0.90%
CBN	1.25	1.21	0.01	97%	1.10%	1.19	0.02	95%	1.30%	1.24	0.01	99%	1.20%	1.25	0.01	100%	1.00%	1.25	0.01	100%	1.10%
	2.5	2.51	0.06	100%	2.30%	2.46	0.06	98%	2.30%	2.49	0.04	100%	1.80%	2.51	0.06	100%	2.50%	2.52	0.06	101%	2.40%
	5	5.15	0.03	103%	0.60%	5.1	0.03	102%	0.60%	5.16	0.04	103%	0.80%	5.15	0.05	103%	0.90%	5.19	0.05	104%	1.00%
CBG	1.25	1.23	0.06	98%	5.30%	1.19	0.02	95%	1.30%	1.2	0.02	96%	1.30%	1.23	0.01	98%	1.10%	1.21	0.01	97%	1.10%
	2.5	2.42	0.07	97%	3.00%	2.45	0.06	98%	2.50%	2.48	0.04	99%	1.60%	2.52	0.06	101%	2.20%	2.52	0.07	101%	2.60%
	5	4.97	0.12	99%	2.50%	5.12	0.03	102%	0.60%	5.24	0.03	105%	0.60%	5.27	0.04	105%	0.80%	5.29	0.05	106%	0.90%
CBE	1.25	1.24	0.08	99%	6.50%	1.16	0.02	93%	1.30%	1.15	0.01	92%	1.00%	1.16	0.01	93%	1.10%	1.17	0.01	93%	1.30%
	2.5	2.62	0.05	105%	1.80%	2.42	0.06	97%	2.40%	2.44	0.04	98%	1.80%	2.47	0.06	99%	2.60%	2.49	0.06	99%	2.50%
	5	5.33	0.1	107%	1.90%	5.09	0.07	102%	1.30%	5.19	0.06	104%	1.20%	5.24	0.06	105%	1.20%	5.29	0.06	106%	1.20%
THCVA	1.25	1.17	0.02	94%	1.7%	1.15	0.01	92%	0.7%	1.17	0.02	93%	1.8%	1.22	0.01	98%	1.0%	1.21	0.02	97%	1.30%
	2.5	2.44	0.06	98%	2.3%	2.41	0.05	97%	2.2%	2.44	0.04	98%	1.7%	2.47	0.05	99%	2.2%	2.46	0.05	98%	2.00%
	5	5.1	0.02	102%	0.4%	5.05	0.03	101%	0.6%	5.17	0.03	103%	0.6%	5.1	0.05	102%	0.9%	5.13	0.04	103%	0.90%
CBDA	1.25	1.19	0.02	95%	1.4%	1.16	0.02	93%	1.5%	1.22	0.01	97%	1.2%	1.22	0.01	98%	1.0%	1.23	0.01	98%	1.10%
	2.5	2.45	0.05	98%	2.1%	2.41	0.06	96%	2.3%	2.44	0.04	97%	1.7%	2.44	0.06	98%	2.3%	2.46	0.06	98%	2.30%
	5	5.1	0.02	102%	0.5%	5.04	0.03	101%	0.6%	5.07	0.03	101%	0.7%	5.05	0.04	101%	0.8%	5.09	0.05	102%	0.90%
CBLA	1.25	1.13	0.02	90%	1.6%	1.28	0.01	102%	1.0%	1.27	0.01	102%	0.9%	1.31	0.01	105%	1.1%	1.3	0.01	104%	1.1%
	2.5	2.45	0.06	98%	2.4%	2.51	0.05	100%	2.2%	2.51	0.04	101%	1.5%	2.55	0.06	102%	2.2%	2.54	0.06	102%	2.3%
	5	5.13	0.02	103%	0.4%	5.05	0.03	101%	0.6%	5.14	0.04	103%	0.7%	5.14	0.04	103%	0.8%	5.16	0.05	103%	0.9%
THCAA	1.25	1.28	0.02	103%	1.50%	1.28	0.02	102%	1.20%	1.25	0.02	100%	1.80%	1.31	0.01	105%	0.70%	1.28	0.02	102%	1.50%
	2.5	2.52	0.06	101%	2.20%	2.49	0.06	100%	2.30%	2.48	0.04	99%	1.50%	2.51	0.07	100%	2.80%	2.51	0.06	100%	2.20%
	5	5.09	0.03	102%	0.60%	5.02	0.04	100%	0.80%	5.13	0.03	103%	0.70%	5.12	0.06	102%	1.20%	5.12	0.04	102%	0.80%

continued

▶ **Table 3** *Continued*

Compound	Concentration (µg/mL)	Batch 1				Batch 2				Batch 3				Batch 4				Batch 5			
		Mean	SD	Trueness	Precision (%RSD)	Mean	SD	Trueness	Precision (%RSD)	Mean	SD	Trueness	Precision (%RSD)	Mean	SD	Trueness	Precision (%RSD)	Mean	SD	Trueness	Precision (%RSD)
CBCA	1.25	1.19	0.02	100%	1.60%	1.2	0.02	101%	1.30%	1.15	0.02	97%	1.50%	1.15	0.01	97%	1.30%	1.15	0.01	97%	1.10%
	2.5	2.48	0.05	100%	2.00%	2.44	0.05	99%	1.90%	2.45	0.03	99%	1.40%	2.45	0.05	99%	2.20%	2.46	0.05	100%	2.20%
	5	5.14	0.03	99%	0.60%	5.04	0.03	97%	0.60%	5.21	0.04	101%	0.80%	5.21	0.05	101%	0.90%	5.25	0.05	101%	1.00%
CBGA	1.25	1.17	0.02	94%	1.40%	1.15	0.01	92%	1.10%	1.15	0.02	92%	1.30%	1.14	0.01	91%	1.30%	1.25	0.02	100%	1.30%
	2.5	2.44	0.05	98%	2.10%	2.4	0.05	96%	2.30%	2.43	0.04	97%	1.80%	2.45	0.06	98%	2.60%	2.49	0.06	100%	2.40%
	5	5.08	0.02	102%	0.40%	5.03	0.03	101%	0.50%	5.16	0.04	103%	0.90%	5.18	0.05	104%	1.00%	5.12	0.06	102%	1.20%
CBEA	1.25	1.26	0.04	101%	3.00%	1.24	0.01	99%	1.00%	1.22	0.01	98%	1.10%	1.23	0.01	99%	1.20%	1.24	0.01	99%	1.20%
	2.5	2.55	0.06	102%	2.20%	2.44	0.05	98%	2.10%	2.48	0.04	99%	1.50%	2.49	0.06	100%	2.40%	2.5	0.06	100%	2.40%
	5	5.14	0.06	103%	1.20%	4.96	0.03	99%	0.50%	5.2	0.05	104%	1.00%	5.16	0.06	103%	1.20%	5.21	0.07	104%	1.30%
CBNA	1.25	1.24	0.02	99%	1.40%	1.22	0.01	98%	1.10%	1.22	0.01	98%	1.10%	1.23	0.01	98%	1.10%	1.23	0.01	98%	1.10%
	2.5	2.48	0.05	99%	2.20%	2.46	0.05	98%	2.10%	2.47	0.04	99%	1.60%	2.48	0.05	99%	2.20%	2.48	0.05	99%	2.20%
	5	5.07	0.03	101%	0.50%	5.05	0.03	101%	0.60%	5.15	0.04	103%	0.80%	5.14	0.04	103%	0.90%	5.15	0.05	103%	1.00%

▶ **Table 4** Inter-day precision and accuracy (n = 30).

Cannabinoid	Concentration (µg/mL)	Between Batches			
		Mean	SD	Trueness	Precision (%RSD)
CBDV	1.25	1.22	0.02	99%	1.7%
	2.5	2.47	0.04	100%	1.8%
	5	5.09	0.12	101%	2.4%
THCV	1.25	1.22	0.03	98%	2.1%
	2.5	2.49	0.02	100%	0.8%
	5	5.16	0.04	103%	0.9%
CBT	1.25	1.21	0.03	97%	2.4%
	2.5	2.48	0.02	99%	0.8%
	5	5.17	0.05	103%	1.0%
CBD	1.25	1.20	0.03	96%	2.2%
	2.5	2.48	0.02	99%	0.6%
	5	5.16	0.05	103%	0.9%
CBL	1.25	1.234	0.04	99%	3.2%
	2.5	2.496	0.02	100%	0.9%
	5	5.166	0.04	103%	0.8%
CBC	1.25	1.2	0.02	99.0%	1.7%
	2.5	2.484	0.02	99.8%	0.6%
	5	5.18	0.07	100.8%	1.4%
Δ^8-THC	1.25	1.22	0.03	98%	2.1%
	2.5	2.49	0.01	100%	0.5%
	5	5.15	0.08	103%	1.5%
Δ^9-THC	1.25	1.24	0.02	99%	1.7%
	2.5	2.51	0.02	100%	0.9%
	5	5.19	0.04	104%	0.8%
CBDVA	1.25	1.16	0.03	93%	2.9%
	2.5	2.41	0.03	96%	1.1%
	5	5.11	0.09	102%	1.7%
CBN	1.25	1.23	0.027	98%	2.2%
	2.5	2.50	0.024	100%	1.0%
	5	5.15	0.032	103%	0.6%
CBG	1.25	1.21	0.018	97%	1.5%
	2.5	2.48	0.044	99%	1.8%
	5	5.18	0.134	103%	2.6%
CBE	1.25	1.18	0.036	94%	3.1%
	2.5	2.49	0.079	100%	3.2%
	5	5.23	0.093	105%	1.8%
THCVA	1.25	1.18	0.03	95%	2.5%
	2.5	2.44	0.02	98%	0.9%
	5	5.11	0.04	102%	0.9%

continued

▶ **Table 4** *Continued*

Cannabinoid	Concentration (µg/mL)	Between Batches			
		Mean	SD	Trueness	Precision (%RSD)
CBDA	1.25	1.20	0.03	96%	2.4%
	2.5	2.44	0.02	97%	0.8%
	5	5.07	0.03	101%	0.5%
CBLA	1.25	1.26	0.07	101%	5.8%
	2.5	2.51	0.04	101%	1.6%
	5	5.12	0.04	103%	0.8%
THCAA	1.25	1.28	0.02	102%	1.7%
	2.5	2.502	0.02	100%	0.7%
	5	5.096	0.05	102%	0.9%
CBCA	1.25	1.17	0.02	98%	2.1%
	2.5	2.46	0.02	99%	0.6%
	5	5.17	0.08	100%	1.6%
CBGA	1.25	1.17	0.04	94%	3.8%
	2.5	2.44	0.03	98%	1.3%
	5	5.11	0.06	102%	1.2%
CBEA	1.25	1.24	0.01	99%	1.2%
	2.5	2.49	0.04	100%	1.6%
	5	5.13	0.10	103%	2.0%
CBNA	1.25	1.23	0.01	98%	0.7%
	2.5	2.47	0.01	99%	0.4%
	5	5.11	0.05	102%	0.9%

mixture). Then, 50 µL of I.S and 10 µL of a 2% DMAP solution were added to each concentration of the cannabinoid mixture. Under a flow of nitrogen gas at 50 °C, the points were evaporated to dryness in GC vials. Next, the residue obtained was derivatized by adding 100 µL of BSTFA, the vials were capped and placed in an oven set at 70 °C for 30 min. Afterward, the vials were cooled to room temperature, the contents transferred to inserts, and 2 µL injections were made into the GC-FID. Calibration curves were obtained in six replicates and constructed by plotting the concentration versus peak area ratio (peak area of analyte/peak area of I. S.). Quality control samples independently prepared at three different concentrations (low, medium, and high) for each cannabinoid were prepared in the same way every day and on five consecutive days (one batch every day). All cannabinoid standards, stock solutions, and QC samples were stored at − 20 °C till the time of analysis.

Preparation of extracts of *C. sativa* plant materials

Samples from cannabis buds were separately dried in a ventilated oven at 40 °C for 24 h and then powdered. Triplicates of the ground samples (100 mg each) were weighed into a centrifuge tube and each extracted with 10 mL of a MeCN : MeOH mixture (8 : 2) by sonication for 30 min. The mixture was centrifuged for 5 min. at 1252 xg and transferred into pre-labeled extraction tubes. Aliquots of 10 µL, 50 µL, and 100 µL were transferred into pre-labeled GC vials. To each vial, 50 µL of 1 mg/mL I. S. solution and 10 µL of 2% DMAP were added and the solvents evaporated to dryness using a gentle flow of nitrogen gas at 50 °C. The residue was then silylated by adding 100 µL of BSTFA, vortexed, and the capped vials were kept in a 70 °C oven for 30 min. The vials were then brought to room temperature and the contents were transferred to 100 µL GC-vial inserts and analyzed by the GC-FID. The injection volume was 2 µL.

Method validation

The GC-FID method validation included linearity, selectivity, the limit of detection (LOD), the limit of quantification (LOQ), trueness, and precision and was performed according to the International Conference on Harmonization (ICH) Tripartite Guideline for Validation of Analytical Procedures [46]. Trueness was measured by the standard addition method. The intra-day and inter-day were assessed using a series of measurements. Six-point standard calibration curves were used to evaluate linearity. Calibration graphs were constructed by plotting the peak area ratio (y) of

▶ **Table 5** Calculated concentrations from different *Cannabis sativa* chemovars (% w/w).

Variety	High THC Chemovar (R0466) 1606-THC	Intermediate Chemovar (R0466) 1326-IM	Intermediate Chemovar (R0466) B5-1597-IM	High CBD Chemovar (R0466) 1594-CBD
CBDV	ND	ND	ND	0.04
THCV	ND	ND	ND	ND
CBT	ND	ND	ND	ND
CBD	ND	0.61	1.04	2.13
CBL	ND	ND	ND	ND
CBC	ND	0.08	0.06	0.18
Δ^8-THC	bLOQ	ND	0.05	ND
Δ^9-THC	1.11	0.55	0.66	0.07
CBDVA	ND	0.03	0.05	0.19
CBG	bLOQ	0.03	0.06	0.04
CBN	bLOQ	ND	ND	ND
CBE	ND	ND	ND	ND
THCVA	0.08	0.05	0.04	ND
CBDA	ND	6.50	12.74	11.44
CBLA	ND	0.09	0.06	ND
THCAA	18.96	5.12	6.80	0.33
CBCA	0.22	0.35	0.55	0.52
CBGA	0.49	0.41	0.63	ND
CBEA	ND	ND	ND	ND
CBNA	0.12	0.15	0.18	ND

bLOQ = below Limit of Quantitation. ND = not detected.

each analyte to that of IS versus the analyte concentration (x) by injecting triplicates of each concentration. Linear regression with a 1/x weighting factor described the regression relationship. Linearity was considered satisfactory if the correlation coefficient (R^2) of the calibration was higher than 0.99.

LOD and LOQ were determined as LOD = 3.3σ/S and LOQ = 10σ/S, where σ = standard deviation of the response of each cannabinoid and S = slope of the calibration curve of each cannabinoid.

To verify method precision, the relative standard deviation (% RSD) of each batch (intra-day precision) was calculated for five consecutive days (n = 6) and between batches (inter-day precision) (n = 30). Trueness was calculated as % recovery and precision (stated as RSD%). The intra-day and inter-day precisions were required to be equal to or less than 15%, and the trueness to be within ± 15% recovery.

Contributors' Statement

Data collection: I. Shahzadi, S. W. Gul, E. A. Ibrahim, S. Chandra, H. Lata; design of the study: W. Gul, M. A. ElSohly, M. M. Radwan, E. A. Ibrahim, I. Shahzadi; statistical analysis: I. Shahzadi, S. W. Gul, E. A. Ibrahim; analysis and interpretation of the data: E. A. Ibrahim, S. W. Gul, I. Shahzadi, S. Chandra, H. Lata, M. M. Radwan; drafting the manuscript: E. A. Ibrahim, S. W. Gul, M. M. Radwan, W. Gul; critical revision of the manuscript: M. A. ElSohly, W. Gul, M. M. Radwan.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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