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Analysis of Terpenes in *Cannabis sativa* L. Using GC/MS: Method Development, Validation, and Application

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Key words

Cannabis sativa, Cannabaceae, terpenes, GC-MS, method development, method validation

received May 15, 2018
revised December 4, 2018
accepted December 18, 2018

Bibliography

DOI https://doi.org/10.1055/a-0828-8387
Published online January 15, 2019 | Planta Med 2019; 85: 431–438 © Georg Thieme Verlag KG Stuttgart · New York | ISSN 0032-0943

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ABSTRACT

Terpenes are the major components of the essential oils present in various *Cannabis sativa* L. varieties. These compounds are responsible for the distinctive aromas and flavors. Besides the quantification of the cannabinoids, determination of the terpenes in *C. sativa* strains could be of importance for the plant selection process. At the University of Mississippi, a GC-MS method has been developed and validated for the quantification of terpenes in cannabis plant material, viz., α-pinene, β-pinene, β-myrcene, limonene, terpinolene, linalool, α-terpineol, β-caryophyllene, α-humulene, and caryophyllene oxide. The method was optimized and fully validated according to AOAC (Association of Official Analytical Chemists) guidelines against reference standards of selected terpenes. Samples were prepared by extraction of the plant material with ethyl acetate containing *n*-tridecane solution (100 µg/mL) as the internal standard. The concentration-response relationship for all analyzed terpenes using the developed method was linear with r^2 values > 0.99. The average recoveries for all terpenes in spiked indoor cultivated samples were between 95.0–105.7%, with the exception of terpinolene (67–70%). The measured repeatability and intermediate precisions (% relative standard deviation) in all varieties ranged from 0.32 to 8.47%. The limit of detection and limit of quantitation for all targeted terpenes were determined to be 0.25 and 0.75 µg/mL, respectively. The proposed method is highly selective, reliable, and accurate and has been applied to the simultaneous determination of these major terpenes in the *C. sativa* biomass produced by our facility at the University of Mississippi as well as in confiscated marijuana samples.

ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
CBD	cannabidiol
GC-FID	gas chromatography coupled with a flame ionization detector
HD	high cannabidiol cannabis variety
HP	high potency (high *trans*-Δ^9-tetrahydrocannabinol) cannabis variety
HS	headspace
IM	intermediate cannabis variety
IS	internal standard
LOD	limit of detection
LOQ	limit of quantitation
RSD	relative standard deviation
THC	*trans*-Δ^9-tetrahydrocannabinol

Introduction

Cannabis sativa L. (family Cannabaceae) is the most frequently used illicit plant (marijuana or hashish) and is considered a valuable medicinal plant with a variety of therapeutic benefits [1,2]. A few cannabis-based medicines in the form of pure cannabinoids or cannabis extracts are now available on the pharmaceutical market [3–6] to treat different medical conditions such as cancer chemotherapy-induced nausea and vomiting, appetite loss, and cachexia in cancer, AIDS, and HIV patients [7–10], neuropathic and chronic pain, and spasticity in multiple sclerosis [10,11].

Several chemical constituents have been identified in *C. sativa* including cannabinoids, terpenes, and phenolic compounds [12]. More than 120 cannabinoids and 120 terpenes (mainly mono- and sesquiterpenes) have been recognized in *C. sativa* [13–15]. Δ^9-THC is considered the main psychoactive cannabinoid, while CBD is nonpsychoactive, but known to be important, for example, in the treatment of epilepsy [16–18]. A standardized cannabis extract containing a combination of THC/CBD at a ratio 1:1 was developed by GW Pharma for the treatment of serious muscle strains, spasticity, and multiple sclerosis [16,19,20]. Terpenes are produced in cannabis trichomes along with the cannabinoids, and are responsible for the plant's characteristic smell [15]. Only a few reports have been published on the possible contribution of terpenes to the activity of cannabis. For example, pinene has been reported as an acetylcholinesterase inhibitor aiding memory, which may counteract THC intoxication side effects [21,22]. The sesquiterpene β-caryophyllene (reaching 2 mg/g), the most predominant sesquiterpene found in cannabis, was shown to interact with cannabinoid receptor type 2, and be responsible for the anti-inflammatory effects of some cannabis preparations [23,24]. Interestingly, caryophyllene oxide has been reported as the main component responsible for cannabis identification by drug-sniffing dogs [25]. Much more research on cannabis terpenes' pharmacology, synergism, and mechanism of action is therefore needed to fully understand the contribution of terpenes in the activity of cannabis.

GC-FID and GC/MS are the most frequently used methods for the analysis of volatile terpenes [26–30]. Other gas chromatography techniques such as HS GC/FID, HS GC/MS, two-dimensional (GC × GC/qMS), HS solid-phase microextraction (HS-SPME), GC/MS, and GC × GC/MS were also used for the analysis of cannabis and hashish samples [31–33]. Romano and Hazekamp compared five different methods for cannabis oil preparation (using naphtha, petroleum ether, ethanol, and two types of olive oil). They performed the analysis of both terpenes and cannabinoids using GC-FID and HPLC-UV, respectively [34]. Recently, a GC/MS method was developed by Sexton et al. to quantify terpenes in cannabis flowers and supercritical fluid CO_2 (SC-CO_2) extract of materials grown in Washington State [35]. It is worth mentioning that none of the current cannabis classification systems are based on terpenes [36]. For quality control of the cannabis samples, it would therefore be important to consider not only cannabinoids, but also other constituents, such as terpenes [37].

This report deals the development and full validation of a simple and precise GC/MS analytical method for accurate and efficient determination of the major cannabis terpenes, namely, the monoterpenes, α-pinene, β -pinene, β-myrcene, limonene, linalool, α-terpineol, and terpinolene and the sesquiterpenes, β-caryophyllene, α-humulene, and caryophyllene oxide. To our knowledge, this is the first GC/MS method validated for quantification of the ten most abundant terpenes in cannabis.

Results and Discussion

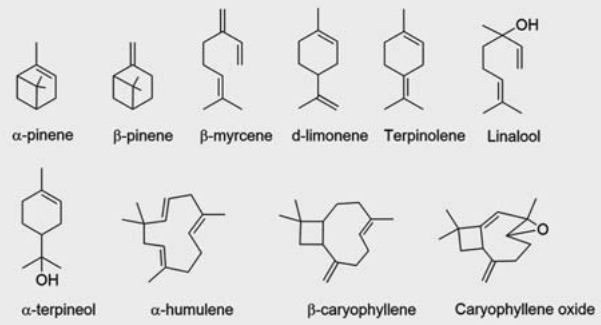
A GC/MS method was developed and validated following the AOAC guidelines [38] for the identification and quantification of the prevalent terpenes in *C. sativa*, as shown in ▶ **Fig. 1**. The method was optimized to achieve the best separation with satisfactory retention times.

n-Tridecane (C_{13}) was selected as the IS since it was found experimentally that its retention time falls between the mono- (C_{10}) and sesquiterpenes (C_{15}), and it was not present in *C. sativa* plant extracts.

Different extraction solvents were tested to produce the highest extraction efficiency, including ethyl acetate, ethanol, methanol, and a chloroform : methanol (1 : 9) mixture. Ethyl acetate gave the best recovery results compared to the other solvents.

Two different GC columns, DB-1MS and DB-5MS, with different dimensions were evaluated. DB-5MS (30 m × 0.25 mm i. d. × 0.25 µm film thickness) was selected because this column provided the best baseline separation for the tested terpenes. The temperature program was optimized to achieve the optimum separation between all the target terpenes without any interference with any components of the plant matrix. Terpenes in samples were identified based on comparison of their retention times and spectral data with those of the reference standards. Representative chromatograms of the standard terpene mixture (▶ **Fig. 2A**), as well as chromatograms representing the HP type, the THC/CBD type (IM), and the CBD (HD) type cannabis samples are shown in ▶ **Fig. 2B–D**, respectively.

The LOQ and LOD for each individual terpene were determined to be 0.75 and 0.25 µg/mL, respectively (**Table 1S**, Supporting Information). The calibration curves were linear between 0.75–



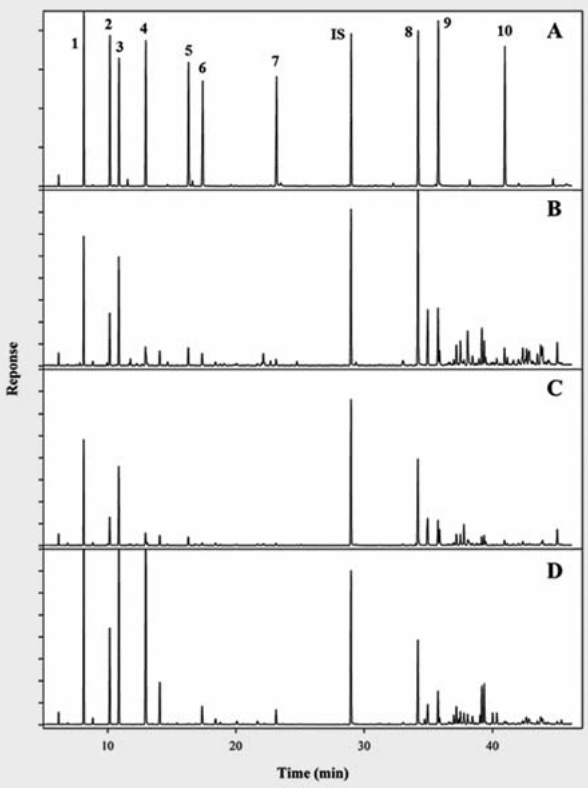
▶ **Fig. 1** Chemical structures of the ten quantified terpenes.

75 µg/mL for *β*-myrcene, *α*-pinene, *β*-pinene, terpinolene, and linalool; 0.75–70 µg/mL for limonene, *α*-terpineol, *β*-caryophyllene, and *α*-humulene, and 1.0–50 µg/mL for caryophyllene oxide, with r^2 values > 0.99 for all terpenes (**Fig. 1S** and **Table 2S**, Supporting Information). Based on our method, the peaks of the analyzed terpenes showed good resolution (> 2), as presented in ▶ **Fig. 2A**. Baseline separation was achieved for all of the terpenes.

The average recoveries for individual terpenes spiked in the placebo marijuana samples were 97.3% for *α*-pinene, 97.0% for *β*-pinene, 98.0% for *β*-myrcene, 98.6% for limonene, 103.0% for linalool, 70.0% for terpinolene, 100.0% for *α*-terpineol, 105.7% for *β*-caryophyllene, 102.0% for *α*-humulene, and 103.7% for caryophyllene oxide (**Table 3S**, Supporting Information). The average recovery from the indoor cultivated samples was 101.7% for *α*-pinene, 97.3% for *β*-pinene, 96.0% for *β*-myrcene, 95.0% for limonene, 95.0% for linalool, 67.0% for terpinolene, 100.0% for *α*-terpineol, 103.3% for *β*-caryophyllene, 102.0% for *α*-humulene, and 96.3% for caryophyllene oxide (**Table 4S**, Supporting Information). To determine the degree of carryover, one ethyl acetate blank was injected after each run (calibration standards or sample solutions). The blank did not show peaks for the analytes or the IS at signal-to-noise ratio of ≥ 3.

The repeatability and intermediate precision were determined in terms of %RSD as shown in **Tables 5S**–**10S**, Supporting Information. The intra- and inter-day precision were found to be less than 15%. For the high CBD variety sample, the measured repeatability was 0.32–5.89% and the intermediate precision was 0.50–6.01% (**Tables 5S** and **6S**, Supporting Information). For the high THC variety samples, the repeatability and the intermediate precision ranged from 0.37–8.47% and 1.47–7.07%, respectively (**Tables 7S** and **8S**, Supporting Information). The repeatability and intermediate precision were also measured for intermediate variety samples and ranged from 0.42–4% and 3.43–6.79%, respectively (**Tables 9S** and **10S**, Supporting Information). All values of repeatability and intermediate precision were within an acceptable range of < 10% and the method was found to be precise (**Tables 5S**–**10S**, Supporting Information).

The developed GC-MS method was applied to the analysis of indoor and outdoor grown plant materials as well as seized samples that were previously analyzed for their cannabinoid content using our previously published GC/FID method [39].



▶ **Fig. 2** Representative total ion chromatogram of the standard terpenes (**A**), sample MX1 (**B**, high THC variety), sample V19 (**C**, high CBD variety), and sample B4 (**D**, intermediate variety). Compound identification is consistent with ▶ **Fig. 1**.

The indoor and outdoor grown samples as well as the seized samples were classified based on their cannabinoid content into three major varieties, high THC or high potency (HP) variety with THC >> CBD, high CBD variety (HD) with CBD >> THC, and intermediate variety (IM) with significant levels of both THC and CBD.

A variation in the total and individual terpene content between different varieties was observed (▶ **Tables 1** and **2**). Within the indoor grown biomass, the content of monoterpenes was higher in the IM variety compared to HD and HP varieties. For example, the content of *α*-pinene was around three times higher in the IM variety (1.41–1.55 mg/g) compared to the HD variety (0.31–0.59 mg/g) or the HP variety (0.21–0.58 mg/g) (▶ **Table 1**). The amount of limonene observed in the IM variety (1.44–1.61 mg/g) was more than tenfold higher compared with the amount in the HP (< 0.15 mg/g) and HD (< 0.09 mg/g) varieties. A similar observation was made for myrcene: its content in the IM variety was 0.87–1.32 mg/g compared to 0.54–0.68 mg/mL and 0.19–0.72 mg/g in HD and HP varieties, respectively. This is an interesting observation knowing that myrcene is thought to positively interact with THC, extending its psychoactive effects [40]. These observations may help in the selection of a specific variety or to differentiate one variety from another on the basis of terpene content.

▲ **Table 1** Quantitative analysis of indoor and outdoor grown plant samples in mg/g (mean ± SD, n = 3).

Plant samples	Variety	α-Pinene	β-Pinene	β-Myrcene	Limonene	Linalool	α-Terpineol	β-Caryophyllene	α-Humulene	Caryophyllene oxide
Indoor grown	IM1	1.410 ± 0.043	0.546 ± 0.014	1.316 ± 0.042	1.530 ± 0.037	0.169 ± 0.004	0.126 ± 0.001	0.506 ± 0.004	0.189 ± 0.002	0.017 ± 0.001
	IM2	1.558 ± 0.002	0.521 ± 0.004	1.279 ± 0.003	1.613 ± 0.011	0.155 ± 0.003	0.124 ± 0.002	0.654 ± 0.001	0.238 ± 0.004	0.033 ± 0.001
	IM3	1.493 ± 0.052	0.513 ± 0.054	0.875 ± 0.084	1.444 ± 0.043	0.156 ± 0.019	0.134 ± 0.018	0.745 ± 0.098	0.256 ± 0.033	0.051 ± 0.006
	HD1	0.493 ± 0.015	0.162 ± 0.005	0.537 ± 0.032	0.086 ± 0.004	0.025 ± 0.001	0.020 ± 0.001	0.554 ± 0.009	0.147 ± 0.003	0.038 ± 0.002
	HD2	0.319 ± 0.003	0.160 ± 0.003	0.556 ± 0.029	0.074 ± 0.003	0.016 ± 0.002	0.012 ± 0.001	0.446 ± 0.016	0.119 ± 0.003	0.023 ± 0.001
	HD3	0.599 ± 0.013	0.179 ± 0.003	0.676 ± 0.011	0.084 ± 0.002	0.013 ± 0.001	0.013 ± 0.001	0.576 ± 0.019	0.157 ± 0.002	0.019 ± 0.001
	HP1	0.583 ± 0.013	0.285 ± 0.005	0.719 ± 0.022	0.154 ± 0.004	0.116 ± 0.001	0.053 ± 0.001	1.262 ± 0.033	0.329 ± 0.011	0.122 ± 0.001
	HP2	0.214 ± 0.009	0.092 ± 0.001	0.186 ± 0.008	0.068 ± 0.001	0.040 ± 0.001	0.020 ± 0.001	0.461 ± 0.006	0.124 ± 0.003	0.040 ± 0.001
	HP3	0.262 ± 0.008	0.151 ± 0.005	0.428 ± 0.016	0.094 ± 0.003	0.067 ± 0.003	0.032 ± 0.002	0.514 ± 0.009	0.138 ± 0.003	0.045 ± 0.001
Outdoor grown	HP4	0.025 ± 0.001	ND	ND	ND	0.119 ± 0.001	0.109 ± 0.001	1.287 ± 0.016	0.404 ± 0.005	0.843 ± 0.004
	HP5	0.033 ± 0.001	0.019 ± 0.001	bLOQ	ND	0.016 ± 0.006	0.010 ± 0.004	0.939 ± 0.029	0.273 ± 0.015	0.221 ± 0.029
	HP6	0.031 ± 0.001	0.019 ± 0.001	bLOQ	ND	0.017 ± 0.007	0.010 ± 0.006	0.936 ± 0.029	0.270 ± 0.015	0.220 ± 0.022
	HD4	0.002 ± 0.002	0.028 ± 0.001	0.014 ± 0.002	0.043 ± 0.001	ND	0.013 ± 0.001	0.586 ± 0.001	0.176 ± 0.003	0.188 ± 0.007

IM = intermediate variety, HD = high CBD variety, HP = high THC variety, ND = not detected, bLOQ = below the limit of quantitation

In all indoor grown plants (harvested in 2017), the overall monoterpene content was moderately higher compared to the outdoor grown plants (harvested in 2014). The two monoterpenes β-myrcene and limonene were even not detected in most of the outdoor grown samples. This may be due, however, to the longer storage period for the outdoor grown plants in accordance with the fact that monoterpenes tend to evaporate more readily than sesquiterpenes during storage of the plant material. The amount of each sesquiterpene was very similar in all samples and independent of the sample's origin. This resulted in a moderately higher ratio of sesquiterpenes compared to monoterpenes in the outdoor grown samples (▶ **Table 1**). The amount of α-humulene was present in all cannabis varieties, and no relationship could be drawn between the amount of α-humulene and the type of cannabis.

The consistency in the terpene content was only noticed in materials grown under the same conditions. There was no consistency or general observation concerning the terpene content of confiscated samples.

More studies are underway using principle component analysis to investigate the distribution of terpenes among different cannabis varieties produced in different states with medical marijuana laws.

Materials and Methods

Standards and reagents

All of the reference standards were purchased from Sigma-Aldrich: α-pinene (purity ≥ 98 %), (−)-β-pinene (purity ≥ 99 %), myrcene (purity ≥ 95 %), (R)-(+)-limonene (purity ≥ 97 %), terpinolene (purity ≥ 85 %), linalool (purity ≥ 97 %), terpineol (purity ≥ 90 %), β-caryophyllene (purity ≥ 80 %), α-humulene (purity ≥ 96 %), caryophyllene oxide (purity ≥ 99 %), and *n*-tridecane (purity ≥ 99 %). Their purities were confirmed by GC/MS prior to the quantification. All solvents and reagents were purchased from Thermo Fisher Scientific and were of HPLC grade.

Cannabis plant material

The indoor and outdoor *C. sativa* plants of three varieties (high THC, THC/CBD, and high CBD) were grown at the University of Mississippi. Outdoor grown plants were harvested in 2014 and indoor grown plants in 2017. These materials as well as a select number of DEA seized plant samples, received by our laboratory for potency monitoring analysis, were analyzed in this study. Voucher specimens from each variety were kept at the Coy Waller Laboratory, University of Mississippi with codes CFP-MX, CFP-V1, and CFP-B4 for high THC, THC/CBD, and high CBD respectively.

GC/MS analysis

Chromatographic conditions

The analyses were performed with an Agilent 7890A series (Agilent) GC equipped with an Agilent 5975C MDS mass detector and an Agilent 7693 autosampler. The column used was a DB-5MS capillary column (30 m × 0.25 mm I. D., 0.25 µm film thickness; Agilent). Helium was used as the carrier gas with a constant flow mode at a flow rate of 1 mL/min. The inlet temperature was

▶ **Table 2** Quantitative analysis of seized plant samples in mg/g (n = 3).

Variety	α-Pinene	β-Pinene	β-Myrcene	Limonene	Linalool	α-Terpineol	β-Caryophyllene	α-Humulene	Caryophyllene oxide
IM 4	0.025 ± 0.001	0.004 ± 0.001	0.034 ± 0.001	0.013 ± 0.001	0.078 ± 0.003	0.114 ± 0.001	0.282 ± 0.009	0.095 ± 0.005	0.410 ± 0.021
IM 5	0.005 ± 0.001	bLOQ	0.008 ± 0.001	ND	0.003 ± 0.005	0.020 ± 0.001	0.394 ± 0.006	0.164 ± 0.001	0.691 ± 0.001
IM 6	0.027 ± 0.001	0.044 ± 0.002	0.036 ± 0.001	0.210 ± 0.001	0.432 ± 0.002	0.208 ± 0.001	1.010 ± 0.017	0.333 ± 0.004	ND
IM 7	0.021 ± 0.001	0.001 ± 0.001	0.029 ± 0.001	0.006 ± 0.001	0.191 ± 0.008	0.189 ± 0.008	0.127 ± 0.001	0.053 ± 0.002	0.230 ± 0.012
IM 8	0.037 ± 0.014	0.015 ± 0.004	0.080 ± 0.001	0.107 ± 0.005	0.101 ± 0.007	0.106 ± 0.009	0.896 ± 0.026	0.334 ± 0.007	0.257 ± 0.014
IM 9	0.206 ± 0.066	0.043 ± 0.001	0.126 ± 0.016	0.022 ± 0.004	0.129 ± 0.007	0.063 ± 0.007	0.865 ± 0.052	0.265 ± 0.021	0.230 ± 0.013
IM 10	0.053 ± 0.007	0.013 ± 0.002	0.020 ± 0.001	0.019 ± 0.003	0.041 ± 0.001	0.095 ± 0.003	0.575 ± 0.036	0.182 ± 0.004	0.227 ± 0.006
IM 11	0.085 ± 0.003	0.017 ± 0.001	0.032 ± 0.002	ND	0.024 ± 0.002	0.020 ± 0.001	0.425 ± 0.003	0.136 ± 0.006	0.276 ± 0.004
IM 12	0.043 ± 0.004	0.019 ± 0.001	0.045 ± 0.001	0.049 ± 0.001	0.254 ± 0.008	0.179 ± 0.007	0.697 ± 0.034	0.247 ± 0.005	0.172 ± 0.002
IM 13	0.022 ± 0.007	0.003 ± 0.001	0.005 ± 0.003	bLOQ	0.020 ± 0.002	0.059 ± 0.005	0.499 ± 0.022	0.220 ± 0.008	0.357 ± 0.003
IM 14	0.004 ± 0.002	ND	0.003 ± 0.002	0.028 ± 0.003	0.022 ± 0.002	0.050 ± 0.006	0.380 ± 0.035	0.160 ± 0.013	0.233 ± 0.021
HP7	0.009 ± 0.001	0.012 ± 0.003	0.014 ± 0.001	0.050 ± 0.001	0.273 ± 0.006	0.180 ± 0.001	0.354 ± 0.003	0.133 ± 0.004	0.329 ± 0.009
HP8	0.126 ± 0.001	0.045 ± 0.001	0.164 ± 0.001	0.139 ± 0.001	0.441 ± 0.001	0.214 ± 0.002	0.531 ± 0.003	0.185 ± 0.001	0.514 ± 0.022
HP9	0.020 ± 0.001	0.030 ± 0.002	0.027 ± 0.001	0.603 ± 0.009	0.801 ± 0.002	0.268 ± 0.003	1.704 ± 0.091	0.681 ± 0.063	1.504 ± 0.044
HP10	0.004 ± 0.003	0.005 ± 0.003	0.007 ± 0.003	0.087 ± 0.002	0.689 ± 0.018	0.151 ± 0.002	0.906 ± 0.026	0.397 ± 0.015	1.210 ± 0.009
HP11	0.013 ± 0.002	0.017 ± 0.001	0.019 ± 0.002	0.278 ± 0.008	1.084 ± 0.009	0.474 ± 0.007	0.458 ± 0.015	0.164 ± 0.004	0.450 ± 0.005
HP12	0.015 ± 0.001	0.011 ± 0.001	0.021 ± 0.001	0.044 ± 0.002	0.041 ± 0.002	0.026 ± 0.001	0.923 ± 0.003	0.316 ± 0.001	0.374 ± 0.026
HP13	0.035 ± 0.009	0.013 ± 0.001	0.047 ± 0.002	bLOQ	0.081 ± 0.001	0.012 ± 0.001	0.369 ± 0.007	0.107 ± 0.001	0.198 ± 0.003
HP14	ND	ND	ND	bLOQ	0.004 ± 0.001	0.003 ± 0.001	0.051 ± 0.001	0.017 ± 0.001	0.023 ± 0.001
HP15	0.104 ± 0.005	0.026 ± 0.001	0.136 ± 0.001	0.012 ± 0.001	0.033 ± 0.001	0.060 ± 0.001	0.617 ± 0.001	0.183 ± 0.005	0.391 ± 0.005
HP16	0.019 ± 0.001	0.002 ± 0.001	0.027 ± 0.001	0.003 ± 0.001	0.044 ± 0.003	0.068 ± 0.003	0.411 ± 0.009	0.142 ± 0.004	0.643 ± 0.009
HP17	0.205 ± 0.001	0.073 ± 0.001	0.172 ± 0.009	0.039 ± 0.003	0.045 ± 0.002	0.032 ± 0.002	0.672 ± 0.043	0.254 ± 0.017	0.351 ± 0.021
HP18	0.252 ± 0.012	0.061 ± 0.001	0.266 ± 0.001	0.067 ± 0.003	0.282 ± 0.011	0.221 ± 0.008	0.779 ± 0.043	0.293 ± 0.018	0.668 ± 0.087
HP19	0.132 ± 0.001	0.154 ± 0.006	0.116 ± 0.041	0.169 ± 0.006	0.089 ± 0.004	0.211 ± 0.003	0.396 ± 0.009	0.120 ± 0.007	0.274 ± 0.042
HP20	0.089 ± 0.029	0.041 ± 0.001	0.133 ± 0.034	ND	0.216 ± 0.001	0.259 ± 0.003	0.528 ± 0.009	0.164 ± 0.002	0.405 ± 0.015
HP21	0.111 ± 0.008	0.035 ± 0.007	0.121 ± 0.014	0.072 ± 0.004	0.227 ± 0.004	0.158 ± 0.014	0.413 ± 0.052	0.136 ± 0.001	0.149 ± 0.021
HP22	0.112 ± 0.015	0.015 ± 0.002	0.226 ± 0.007	0.028 ± 0.003	0.065 ± 0.004	0.059 ± 0.002	2.165 ± 0.003	0.863 ± 0.068	1.476 ± 0.018
HP23	0.203 ± 0.075	0.125 ± 0.004	0.294 ± 0.002	bLO Q	1.948 ± 0.068	0.679 ± 0.008	5.872 ± 0.009	2.119 ± 0.042	0.162 ± 0.011

continued

▲ **Table 2** *Continued*

Variety	α-Pinene	β-Pinene	β-Myrcene	Limonene	Linalool	α-Terpineol	β-Caryophyllene	α-Humulene	Caryophyllene oxide
HP24	0.269 ± 0.027	0.136 ± 0.006	0.206 ± 0.008	0.391 ± 0.008	0.280 ± 0.004	0.294 ± 0.011	0.913 ± 0.004	0.269 ± 0.025	0.263 ± 0.036
HP25	0.066 ± 0.002	0.018 ± 0.001	0.118 ± 0.001	0.083 ± 0.007	0.219 ± 0.002	0.215 ± 0.024	0.768 ± 0.007	0.265 ± 0.006	0.303 ± 0.011
HP26	0.087 ± 0.006	0.020 ± 0.002	0.198 ± 0.002	0.059 ± 0.001	0.341 ± 0.008	0.197 ± 0.001	0.903 ± 0.001	0.326 ± 0.014	0.522 ± 0.075
HP27	0.011 ± 0.001	0.024 ± 0.001	0.088 ± 0.001	0.039 ± 0.001	0.062 ± 0.003	0.216 ± 0.002	1.649 ± 0.006	0.536 ± 0.023	0.243 ± 0.013
HP28	0.085 ± 0.001	0.053 ± 0.009	0.157 ± 0.002	0.174 ± 0.003	0.560 ± 0.088	0.302 ± 0.005	1.157 ± 0.026	0.496 ± 0.002	0.140 ± 0.031
HP29	0.639 ± 0.042	0.241 ± 0.003	0.254 ± 0.002	0.153 ± 0.001	0.241 ± 0.015	0.260 ± 0.026	0.659 ± 0.041	0.184 ± 0.017	0.294 ± 0.027
HD5	0.085 ± 0.006	0.019 ± 0.001	0.157 ± 0.002	0.021 ± 0.001	0.093 ± 0.001	0.090 ± 0.013	0.203 ± 0.024	0.069 ± 0.004	0.049 ± 0.006

IM = intermediate variety, HD = high CBD variety, HP = high THC variety, ND = not detected, bLOQ = below the limit of quantitation

250 °C with a split ratio of 15 : 1. The injection volume was 2 µL. The oven temperature program started at 50 °C (held for 2 min), then ramped up to 85 °C at a rate of 2 °C/min, and to 165 °C at 3 °C/min. The post-run temperature was 280 °C for 10 min.

Mass spectrometric conditions

The mass spectrometer was set in full scan mode from 40–450 amu. The ionization energy was 70 eV. The ion source temperature was 230 °C and the quadrupole temperature was 150 °C. The solvent delay was set to 4 min. The transfer line temperature was 280 °C. Software (NIST) was used to assist in compound identification (version 2.0f; Standard Reference Data Program of the National Institute of Standards and Technology, as distributed by Agilent Technologies).

Quantitative analysis

Standard solutions preparation: Stock standard solution of each terpene [α-pinene, (−)-β-pinene, myrcene, (R)- (+)-limonene, terpinolene, linalool, α-terpineol, β-caryophyllene, α-humulene, caryophyllene oxide] was prepared in ethyl acetate. The standard terpenes were mixed and the concentration of each terpene was adjusted to be 1.0 mg/mL from which serial dilutions were made to prepare the individual points of the calibration curves.

Internal standard preparation: n-Tridecane (C_{13} hydrocarbon) was selected as the IS, and its concentration was kept at 100 µg/mL, which was added to all of the calibration and sample solutions.

Calibration curves: Nine calibration points ranging from 0.75–100 µg/mL were prepared from the previously mentioned stock standard solutions (0.75, 1.0, 2.0, 5.0, 10, 25, 50, 70, and 100 µg/mL) and IS. The concentration of the IS at each calibration point was 100 µg/mL. These solutions were used to construct individual terpene calibration curves (**Fig. 1S**, Supporting Information).

Sample solution preparation: Samples from three varieties of *C. sativa* (drug type, intermediate type, and fiber type) were dried for 24 h at 40 °C in a ventilated oven and then ground in a stainless steel coffee grinder. Triplicates (1.0 g each) of the powdered samples were weighed in a 15-mL centrifuge tube and each were extracted with 10 mL of the extraction solution (100 µg/mL of the IS in ethyl acetate) by sonication for 15 min. The mixture was centrifuged for 5 min at 1252 × *g* and the supernatants (without filtration) were used for the GC/MS analysis.

Method validation

The method was validated according to AOAC guidelines with respect to linearity, accuracy (recovery), selectivity, repeatability, and intermediate precision, LOD, and LOQ [38].

Linearity

The nine-point standard calibration curves were used to evaluate linearity. Calibration curves were determined by plotting the peak area ratio (y) (peak area of each terpene to the peak area of the IS) versus the terpene concentration (x). The concentration-response relationship of the present method was required to be linear with r^2 values ≥ 0.99.

Accuracy (recovery)

To determine the accuracy (recovery) of terpenes, triplicates of a stock standard solution of each terpene were spiked to 1 gm of plant material at three different concentration levels: 0.05, 0.25, and 0.50 mg/g. In this study, two plant materials were used, homogenized indoor grown plant material and placebo (cannabis plant material free from terpenes obtained after exhaustive solvent extraction). The plant materials were analyzed before and after spiking according to the above sample preparation method.

The % recovery (accuracy) of each terpene was calculated as:

$$\frac{(\text{Amount after spiking} - \text{Amount before spiking})}{\text{Spiked amount}} \times 100\%$$

Selectivity

The resolution of the terpene peaks in the GC chromatogram represents the selectivity and it is required to be ≥ 2.

Repeatability and intermediate precision

The method precision was evaluated by analysis of the individual terpenes in three different *C. sativa* varieties. The analysis of samples was made in six replicates on three separate days. The intra- and inter-day precisions were required to be less than 15% (% RSD).

Limit of detection and limit of quantification

LOD and LOQ are expressed as LOD = 3.3σ/S and LOQ = 10σ/S, where σ = standard deviation of the response of each terpene and S = slope of the calibration curve of each terpene.

Supporting information

LOD, LOQ and retention times (**Table 1S**), regression data (**Table 2S**), inter- and intraday precision and accuracy parameters (**Tables 5S–10S**), and calibration curves for targeted terpenes with the regression equation and correlation coefficient (r^2) for each curve (**Fig. 1S**) are available as Supporting Information.

Acknowledgements

This research was partially supported by the National Institute on Drug Abuse (N01DA-15-7793) and by the Egyptian government through the joint supervision program (JS-3576).

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Grotenhermen F. Pharmacology of cannabinoids. Neuro Endocrinol Lett 2004; 25: 14–23

[2] Whiting PF, Wolff RF, Deshpande S, Di Nisio M, Duffy S, Hernandez AV, Keurentjes JC, Lang S, Misso K, Ryder S. Cannabinoids for medical use: a systematic review and meta-analysis. JAMA 2015; 313: 2456–2473

[3] Andreae MH, Carter GM, Shaparin N, Suslov K, Ellis RJ, Ware MA, Abrams DI, Prasad H, Wilsey B, Indyk D. Inhaled cannabis for chronic neuropathic pain: a meta-analysis of individual patient data. J Pain 2015; 16: 1221–1232

[4] Blake DR, Robson P, Ho M, Jubb RW, McCabe CS. Preliminary assessment of the efficacy, tolerability and safety of a cannabis-based medicine (Sativex) in the treatment of pain caused by rheumatoid arthritis. Rheumatology 2006; 45: 50–52

[5] Whittle BA, Guy GW, Robson P. Prospects for new cannabis-based prescription medicines. J Cannabis Ther 2001; 1: 183–205

[6] Guy GW, Stott CG. The Development of Sativex® – a natural cannabis-based Medicine. In: Mechoulam R, ed. Cannabinoids as Therapeutics. Basel: Birkhäuser Basel; 2005: 231–263

[7] Davis MP. Cannabinoids for symptom management and cancer therapy: the evidence. J Natl Compr Canc Netw 2016; 14: 915–922

[8] Gerich ME, Isfort RW, Brimhall B, Siegel CA. Medical marijuana for digestive disorders: high time to prescribe. Am J Gastroenterol 2015; 110: 208

[9] Amato L, Minozzi S, Mitrova Z, Parmelli E, Saulle R, Cruciani F, Vecchi S, Davoli M. Systematic review of safeness and therapeutic efficacy of cannabis in patients with multiple sclerosis, neuropathic pain, and in oncological patients treated with chemotherapy. Epidemiol Prev 2017; 41: 279–293

[10] Rudroff T, Honce JM. Cannabis and multiple sclerosis – The way forward. Front Neurol 2017; 8: 299

[11] Lee G, Grovey B, Furnish T, Wallace M. Medical cannabis for neuropathic pain. Curr Pain Headache Rep 2018; 22: 8

[12] Flores-Sanchez IJ, Verpoorte R. Secondary metabolism in cannabis. Phytochem Rev 2008; 7: 615–639

[13] Radwan MM, ElSohly MA, El-Alfy AT, Ahmed SA, Slade D, Husni AS, Manly SP, Wilson L, Seale S, Cutler SJ. Isolation and pharmacological evaluation of minor cannabinoids from high-potency *Cannabis sativa*. J Nat Prod 2015; 78: 1271–1276

[14] Ahmed SA, Ross SA, Slade D, Radwan MM, Khan IA, ElSohly MA. Minor oxygenated cannabinoids from high potency *Cannabis sativa* L. Phytochemistry 2015; 117: 194–199

[15] Pertwee R, ed. Handbook of Cannabis. New York: Oxford University Press; 2014

[16] Tzadok M, Uliel-Siboni S, Linder I, Kramer U, Epstein O, Menascu S, Nissenkorn A, Yosef OB, Hyman E, Granot D. CBD-enriched medical cannabis for intractable pediatric epilepsy: the current Israeli experience. Seizure 2016; 35: 41–44

[17] Cilio MR, Thiele EA, Devinsky O. The case for assessing cannabidiol in epilepsy. Epilepsia 2014; 55: 787–790

[18] Devinsky O, Cilio MR, Cross H, Fernandez-Ruiz J, French J, Hill C, Katz R, Di Marzo V, Jutras-Aswad D, Notcutt WG. Cannabidiol: pharmacology and potential therapeutic role in epilepsy and other neuropsychiatric disorders. Epilepsia 2014; 55: 791–802

[19] Raphael B, Gabet Y. The skeletal endocannabinoid system: clinical and experimental insights. J Basic Clin Physiol Pharmacol 2016; 27: 237–245

[20] Hill KP. Medical marijuana for treatment of chronic pain and other medical and psychiatric problems: a clinical review. JAMA 2015; 313: 2474–2483

[21] Miyazawa M, Yamafuji C. Inhibition of acetylcholinesterase activity by bicyclic monoterpenoids. J Agric Food Chem 2005; 53: 1765–1768

[22] Nissen L, Zatta A, Stefanini I, Grandi S, Sgorbati B, Biavati B, Monti A. Characterization and antimicrobial activity of essential oils of industrial hemp varieties (*Cannabis sativa* L.). Fitoterapia 2010; 81: 413–419

[23] Gertsch J, Leonti M, Raduner S, Racz I, Chen JZ, Xie XQ, Altmann KH, Karsak M, Zimmer A. Beta-caryophyllene is a dietary cannabinoid. Proc Natl Acad Sci U S A 2008; 105: 9099–9104

[24] Klauke AL, Racz I, Pradier B, Markert A, Zimmer AM, Gertsch J, Zimmer A. The cannabinoid CB_2 receptor-selective phytocannabinoid beta-caryophyllene exerts analgesic effects in mouse models of inflammatory and neuropathic pain. Eur Neuropsychopharmacol 2014; 24: 608–620

[25] Russo EB. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. Br J Pharmacol 2011; 163: 1344–1364

[26] Giese MW, Lewis MA, Giese L, Smith KM. Method for the analysis of cannabinoids and terpenes in cannabis. J AOAC Int 2015; 98: 1503–1522

[27] Casano S, Grassi G, Martini V, Michelozzi M. Variations in terpene profiles of different strains of *Cannabis sativa* L. Acta Hortic 2010; 925: 115–121

[28] Ferioli V, Rustichelli C, Pavesi G, Gamberini G. Analytical characterisation of hashish samples. Chromatographia 2000; 52: 39–44

[29] Careri M, Mangia A. Gas Chromatography-Mass Spectrometry Analysis of Flavors and Fragrances. In: Niessen WMA, ed. Current Practice Gas Chromatography-Mass Spectrometry. New York: CRC Press; 2001: 409

[30] Lockwood GB. Techniques for gas chromatography of volatile terpenoids from a range of matrices. J Chromatogr A 2001; 936: 23–31

[31] Omar J, Olivares M, Amigo JM, Etxebarria N. Resolution of co-eluting compounds of *Cannabis sativa* in comprehensive two-dimensional gas chromatography/mass spectrometry detection with multivariate curve resolution-alternating least squares. Talanta 2014; 121: 273–280

[32] Da Porto C, Decorti D, Natolino A. Separation of aroma compounds from industrial hemp inflorescences (*Cannabis sativa* L.) by supercritical CO2 extraction and on-line fractionation. Ind Crops Prod 2014; 58: 99–103

[33] Marchini M, Charvoz C, Dujourdy L, Baldovini N, Filippi JJ. Multidimensional analysis of cannabis volatile constituents: identification of 5, 5-dimethyl-1-vinylbicyclo[2.1.1]hexane as a volatile marker of hashish, the resin of *Cannabis sativa* L. J Chromatogr A 2014; 1370: 200–215

[34] Romano LL, Hazekamp A. Cannabis oil: chemical evaluation of an upcoming cannabis-based medicine. Cannabinoids 2013; 1: 1–11

[35] Sexton M, Shelton K, Haley P, West M. Evaluation of cannabinoid and terpenoid content: Cannabis flower compared to supercritical CO2 concentrate. Planta Med 2018; 84: 234–241

[36] Hazekamp A, Tejkalová K, Papadimitriou S. Cannabis: From cultivar to chemovar II – A metabolomics approach to cannabis classification. Cannabis Cannabinoid Res 2016; 1: 202–215

[37] Elzinga S, Fischedick J, Podkolinski R, Raber JC. Cannabinoids and terpenes as chemotaxonomic markers in cannabis. Nat Prod Chem Res 2015; 3: 2

[38] Howitz W. AOAC Guidelines for single laboratory Validation of chemical Methods for dietary Supplements and Botanicals. Gaithersburg MD USA: AOAC Int; 2002: 1219

[39] Ibrahim EA, Gul W, Gul SW, Stamper BJ, Hadad GM, Salam RAA, Ibrahim AK, Ahmed SA, Chandra S, Lata H. Determination of acid and neutral cannabinoids in extracts of different strains of *Cannabis sativa* using GC-FID. Planta Med 2018; 84: 250–259

[40] McPartland JM, Russo EB. Non-phytocannabinoid Constituents of Cannabis and herbal Synergy. In: Pertwee R. Handbook of Cannabis. New York: Oxford University Press; 2014: 280–295