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Method Development and Validation for Analysis of Selected Terpenes and Terpenoids in Cannabis and Hemp by GC-MS/MS

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ABSTRACT Terpenes are one of the major components present in cannabis and hemp varieties. The therapeutic effect of cannabis on physical health and brain depends on the specific composition of terpenes. The treatment of mood disorders and anxiety disorders was clinically confirmed and necessitated the entourage effect of terpenes coupled with cannabinoids. Besides the quantification of the cannabinoids, the determination of the terpene quantities and proportions in cannabis and hemp plants could be of importance for the plant selection process. In this study, a Triple Quadrupole Mass Spectrometer method has been developed and validated using SRM technique for the quantification of selected 21 terpenes in cannabis plant materials. Samples were prepared by extraction of the plant materials with ethyl acetate followed by sonication and centrifugation. The final sample extract was directly injected into the analytical instrument. The results showed that the linear range was between 0.05 and 5 mg/g with r^2 values higher than 0.99. The average recoveries for all terpenes in spiked samples were between 92.3 and 105.1%, with the exception of geraniol. The measured repeatability (% relative standard deviation) was acceptable and ranged from 1.6 to 13.2%. The limit of detection and limit of quantitation for targeted terpenes were determined to be 0.03 and 0.05 mg/g, respectively. The proposed method is highly selective, reliable, accurate and has been applied to the simultaneous determination of these major terpenes in the cannabis strains developed by our Department of Medical Sciences, in parts of the plant including inflorescences, leaves, stems, and roots for further registration of plant varieties with the Department of Agriculture.

Keyword: Terpenes, Terpenoids, Canabis, Hemp, GS-MS/MS

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Introduction

Terpenes and terpenoids are aromatic hydrocarbon biomolecules produced by a wide variety of plants serving such purposes as attracting insects for pollination and deterring herbivores.[1,2] This phytochemical activity is crucial for the survival of the species. Terpenes are among the more abundant compounds that provide the unique aroma of cannabis and hemp plant materials and extracts.[3] Terpenes are thought to impact the user's experience and preference of satisfaction linked to the fragrance property. Moreover, cannabis is considered a valuable medicinal plant with a variety of therapeutic benefits.[4,5] Cannabis terpenes have also been shown to contribute to physiological responses associated with the use of cannabis-based consumer products[6] and are credited for the so-called "entourage effect". Despite the fact that it is not typically regulated in Thailand, terpene profiles can provide valuable information for product quality. The quality control of incoming raw ingredients as well as finished products across their intended shelf life is important for health-related product manufacturing.

Of all the natural products found in plants, more than 140 terpenes, mainly mono- and sesquiterpenes, have been recognized in cannabis and hemp.[7,8] Among the large number of cannabis terpenoids, only approximately 20 to 30 terpenoids have been reported and used for the chemotyping of cannabis cultivars.[9,10] The concentration of individual terpenes varies by strain, depending on harvest time and drying or storage conditions. By cause of the volatility of terpenes, the concentration levels decrease over time. Thus, different storage conditions should be taken into consideration to result in appropriate products. The drop in terpene amount over time varies for different terpenes.[11] The recent proliferation of new terpene profiling methods can be attributed to the ever-increasing legalization of cannabis use. Terpene analysis can be beneficial for patients and consumers as well as providers and breeders. Due to the uniqueness of terpene profiles, they can be used by cultivators as a "fingerprint" to partially identify the specific strain in question. Cannabis is wind-pollinated, which also contributes to the variability of cannabis metabolites including terpenes. As a result, many cannabis strains lack the level of standardization on genetic and chemotaxonomic to classify the species.[12]

Due to their high volatility, gas chromatographs equipped with FID and MS detector are the most frequently used technique for the analysis of terpenes.[13,14] The most widely used sample preparation method for cannabis terpenoid analysis throughout the literature is liquid solvent extraction[15] thanks to low cost-effectiveness. GC-MS/MS is among the instruments that have been used to provide a much-needed improvement of identification, selectivity, and sensitivity with a specific ion intensity ratio.[16] Also, headspace (HS) GC was selected to recently report a comprehensive profiling of terpenoids in cannabis.[17]

The following study was conducted to address the development and full validation of a simple preparation of samples and a precise GC-MS/MS analytical method for accurate and efficient determination of the 21 major cannabis terpenes shown in Figure 1 that have

been indicated for pharmacological activities.[6] The method was validated for specificity and selectivity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ). We then applied this quantification procedure on a cannabis strain (GG#1) to analyze four parts of plant including inflorescences, leaves, stems, and roots (20 samples for each part) for further registration of plant varieties with the Department of Agriculture.

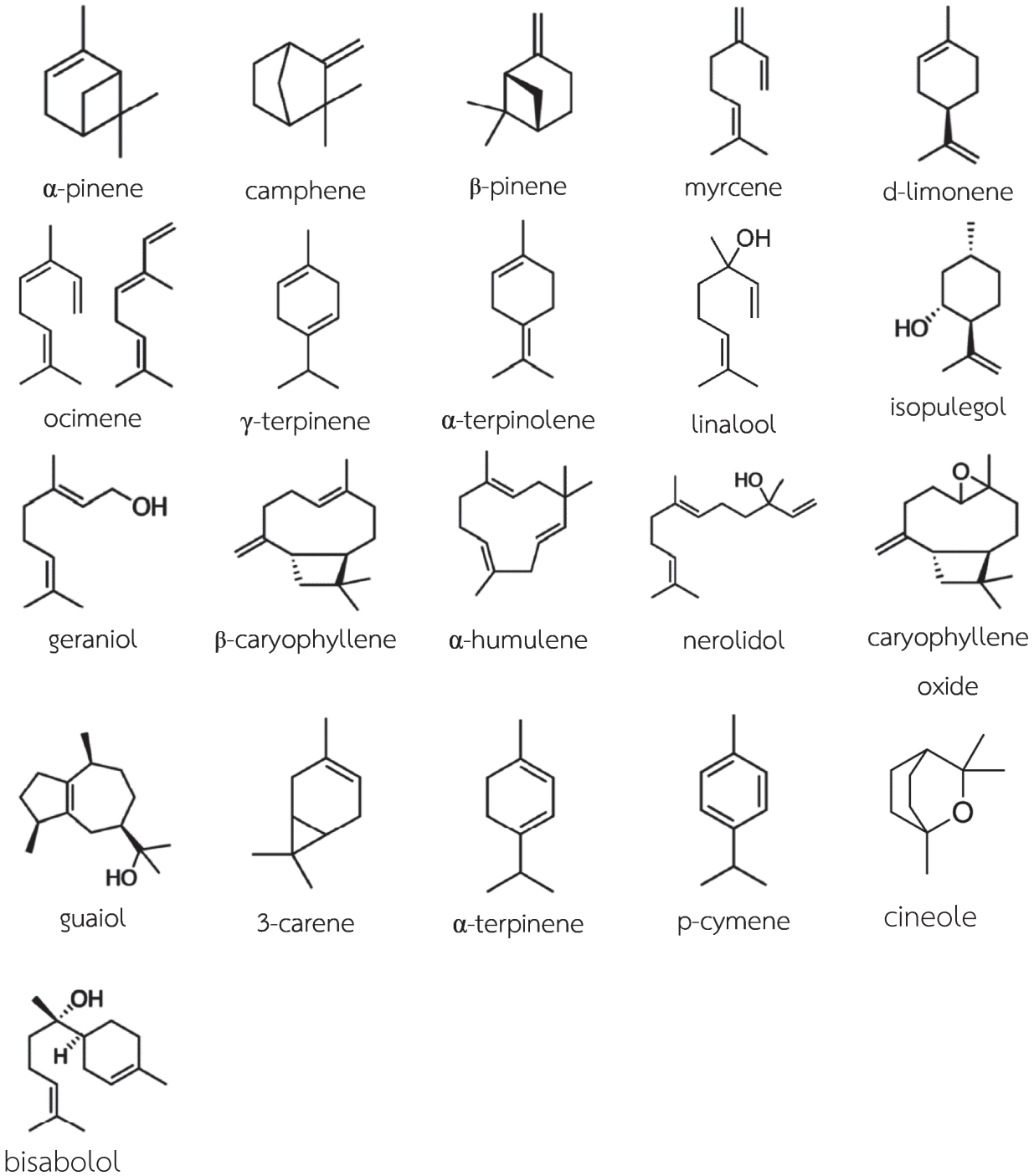

Figure 1 Selected terpenes and terpenoids from naturally occurring cannabis included in this work[16]

Materials and Methods

Chemicals and reagents

Ethyl acetate (HPLC grade) was purchased from J.T. Baker, USA. N-hexane (pesticide grade) and acetone were supplied by RCILabscan, Thailand. Certified reference material, CRM of terpenes standards mixture with certified value and uncertainty approximately 2,500±130 µg/mL were purchased from Dr Ehrenstorfer, USA. The certified value is based on the gravimetric and volumetric preparation of this CRM. Twenty-one terpenes includes α-pinene, camphene, β-pinene, myrcene, d-limonene, ocimene (α-isomer and β-isomer), γ-terpinene, α-terpinolene, linalool, isopulegol, geraniol, β-caryophyllene, α-humulene, nerolidol (isomers 1 and 2), caryophyllene oxide, guaiol, 3-carene, α-terpinene, p-cymene, cineole and bisabolol.

Standard solution preparation

The working standard solutions were prepared by diluting the CRM of terpenes in ethyl acetate to become a working solution of 250 µg/mL and were stored in amber screw-capped glass vials in the dark at -20°C. The calibration curve of standard (1, 5, 10, 20, 50 and 100 µg/mL) was freshly prepared before use by serial dilution of stock solution to the appropriate concentrations. The residue concentrations were calculated using the calibration curve generated from the peak area response versus the working solution concentrations.

Instrument and materials

Digital balances with 1 and 0.01 mg readability for weighing samples and standards were from Sartorius, Germany. The refrigerated centrifuge was Thermo Scientific, a UK product. Vortex mixer was from Vortex-2Genie, USA. Ultrasonic bath cleaner was purchased from Yakos, Taiwan. Calibrated micropipettes, 2-20 µL, 20-200 µL, and 100-1,000 µL, were used for the preparation of the standard solutions. Twenty-milliliter scintillation vials and 4 mL amber vials were used for extraction and reservoir recipient. All glassware were washed with acetone and n-hexane and dried before use.

An analytical method was developed for the Thermo Scientific TRACE™ 1300 Gas Chromatograph and the TSQ 9000, Triple Quadrupole Mass Spectrometer. The GC-MS /MS system was equipped with PAL RTC autosampler using TraceFinder 4.1 EFS software. The analytical conditions of the GC-MS/MS method were provided in Table 1. The MS system was operated in electron ionization mode (EI, 70 eV). The analytes were separated in a fused silica capillary column DB-5MS (30 m x 0.25 mm i.d., 0.25 µm film thickness) from Agilent. The column oven temperatures were programmed as follows: an initial temperature of 50°C was held for 2 min, then increased by 2°C/min to 85°C and then increased by 3°C/min to 165°C and then increased by 30°C/min to 280°C and was held for 10 min. The total run time was approximately 60 min. The injection volume was 2 µL using programmable temperature vaporizing (PTV) as an injector. The PTV temperature was set at 80°C for split injection with a split flow of 50 mL/min. The purge N_2 gas flow was set at 30 mL/min. By using the Thermo AutoSRM software, the two most intense transitions and their optimal

collision energies were selected. The most intense product was selected as the quantifier ion and the second most intense was set as the qualifier ion. Depending on the type of matrix interference, alternative transitions could be selected for quantitative and qualitative ions. The settings on the mass spectrometry detector, including the retention time (RT), quantitative peak, confirmation peak, and collision energies (CE) were reported as shown in Table 1.

Table 1 Selected reaction monitoring (SRM) transitions and collision energies of terpenes

Analyte	Transition type	RT (min)	Window (min)	Mass	Product mass	CE (eV)
α-pinene	Quantification	6.67	0.50	91.10	65.10	16
	Qualification-1	6.67	0.50	93.10	77.00	12
	Qualification-2	6.67	0.50	93.10	91.10	6
camphene	Quantification	7.31	0.50	93.10	77.10	12
	Qualification-1	7.31	0.50	93.10	91.10	8
	Qualification-2	7.31	0.50	121.10	93.10	8
β-pinene	Quantification	8.51	0.50	69.10	41.10	6
	Qualification-1	8.51	0.50	121.10	79.10	10
	Qualification-2	8.51	0.50	121.10	93.10	6
myrcene	Quantification	9.19	0.50	41.10	39.10	8
	Qualification-1	9.19	0.50	69.10	39.10	14
	Qualification-2	9.19	0.50	69.10	41.10	6
3-carene	Quantification	10.02	0.50	79.10	77.10	10
	Qualification-1	10.02	0.50	121.10	93.10	6
	Qualification-2	10.02	0.50	121.10	105.10	6
α-terpinene	Quantification	10.49	0.50	93.10	77.10	10
	Qualification-1	10.49	0.50	93.10	91.10	6
	Qualification-2	10.49	0.50	121.10	93.10	6
p-cymene	Quantification	10.96	0.50	119.10	91.10	12
	Qualification-1	10.96	0.50	119.10	117.10	8
	Qualification-2	10.96	0.50	134.10	119.10	8
d-limonene	Quantification	11.15	0.50	68.10	67.10	6
	Qualification-1	11.15	0.50	93.10	77.10	12
	Qualification-2	11.15	0.50	93.10	91.10	6
cineole	Quantification	11.26	0.50	71.10	43.10	5
	Qualification-1	11.26	0.50	111.10	43.10	10
	Qualification-2	11.26	0.50	111.10	55.10	10
α-ocimene	Quantification	11.66	0.50	79.10	77.10	10
	Qualification-1	11.66	0.50	79.10	77.10	10
	Qualification-2	11.66	0.50	93.10	91.10	6
β-ocimene	Quantification	12.23	0.50	79.10	77.10	10
	Qualification-1	12.23	0.50	79.10	77.10	10
	Qualification-2	12.23	0.50	93.10	91.10	6

Table 1 Selected reaction monitoring (SRM) transitions and collision energies of terpenes (continued)

Analyte	Transition type	RT (min)	Window (min)	Mass	Product mass	CE (eV)
γ-terpinene	Quantification	12.80	0.50	93.10	77.00	12
	Qualification-1	12.80	0.50	93.10	91.10	6
	Qualification-2	12.80	0.50	136.10	93.10	10
α-terpinolene	Quantification	14.36	0.50	121.10	93.10	6
	Qualification-1	14.36	0.50	136.10	93.10	10
	Qualification-2	14.36	0.50	136.10	121.10	8
linalool	Quantification	15.63	1.00	55.10	29.10	10
	Qualification-1	15.63	1.00	71.10	41.10	10
	Qualification-2	15.63	1.00	71.10	43.00	6
isopulegol	Quantification	18.47	1.00	81.10	79.10	6
	Qualification-1	18.47	1.00	121.10	77.10	20
	Qualification-2	18.47	1.00	121.10	93.10	8
geraniol	Quantification	26.10	1.00	41.10	39.10	8
	Qualification-1	26.10	1.00	69.10	39.10	16
	Qualification-2	26.10	1.00	69.10	41.10	6
β-caryophyllene	Quantification	33.04	0.50	93.10	77.00	10
	Qualification-1	33.04	0.50	93.10	91.10	6
	Qualification-2	33.04	0.50	133.10	105.00	8
α-humulene	Quantification	34.72	0.50	93.10	77.00	12
	Qualification-1	34.72	0.50	147.10	77.10	32
	Qualification-2	34.72	0.50	147.10	105.10	8
nerolidol 1	Quantification	38.44	1.00	69.10	41.10	6
	Qualification-1	38.44	1.00	93.10	77.00	12
	Qualification-2	38.44	1.00	107.10	91.10	10
nerolidol 2	Quantification	39.74	1.00	69.10	41.10	6
	Qualification-1	39.74	1.00	93.10	77.00	12
	Qualification-2	39.74	1.00	107.10	91.10	10
caryophyllene	Quantification	40.28	0.50	79.10	51.10	20
oxide	Qualification-1	40.28	0.50	79.10	77.10	10
	Qualification-2	40.28	0.50	93.10	77.10	10
guaiol	Quantification	41.09	1.00	107.10	91.10	10
	Qualification-1	41.09	1.00	161.10	105.10	8
	Qualification-2	41.09	1.00	161.10	119.10	8
bisabolol	Quantification	44.74	1.00	109.10	67.10	8
	Qualification-1	44.74	1.00	119.10	91.10	14
	Qualification-2	44.74	1.00	119.10	117.10	12

Sample and sample preparations

Sample for method development and validation

The sample blank selected is a material known not to contain detectable levels of the terpenes sought for spiking trace level of analytes. A sample blank of dried cannabis leaves (~10% moisture w/w) was obtained from the Medicinal Plant Research Institute, Department of Medical Sciences. Also known as a matrix blank, the sample was homogenized into powder using a cryogenic blender (POWTEQ HM100, China) and passed through a 2-mm sieve as a blank sample to optimize and validate the method. The blank sample was then macerated in ethyl acetate for 30 min and the extract was filtered through the filter paper No.2. The collected powdered cannabis sample was dried in a ventilated oven at 50°C for 24 hours before use in method validation. The samples (0.1 g) from previous step were weighed into 20 mL scintillation vial and spiked with appropriate amount of standard solution. Five milliliters of ethyl acetate was added to the vial and closed suddenly with a screw cap. After mixing by vortex for 1 minute and sonication for 20 minutes, the vial was left at room temperature for at least 10 minutes. Then, approximately 4 mL of the resultant extract was transferred to a 15 mL test tube. Samples were centrifuged at 4,000 rounds per minute for 5 minutes and a portion was transferred to 2 mL GC vials.

Sample analysis for registration of plant varieties

A variety of Thai cannabis, GG#1, was developed by comprehensive research, including the study of botanical characteristics, chemical and genetic profile in the Department of Medical Sciences. The species would be supervised and have distinctly different characteristics. To be registered by the Department of Agriculture, the terpene profile was determined in each variety from four parts of plant including inflorescences, leaves, stems and roots (4 × 20 samples).

Method development and validation

The method was developed and validated in accordance with AOAC guidelines with respect to linearity, accuracy (recovery), selectivity, repeatability, LOD, and LOQ. To achieve high sensitivity, MS conditions were optimized. An appropriate concentration of working solution of mixed standards was prepared and injected into the GC-MS/MS system. The obtained optimum MS parameters are selected. The identification of each compound with corresponding retention time were obtained by comparing with library database of instrument software.

The external standard calibration curve was performed for the quantification step. The six-point standard calibration curves were obtained by spiking mixed standards, ranging from 1 to 100 μg/mL, into ethyl acetate used as a solvent. The concentration-response relationship of the present method was required to be linear with r^2 values ≥ 0.99. Accuracy, expressed as a percentage of recovery and precision (%RSD) was determined based on 10 replicated samples spiked at 0.05, 0.5, and 5 mg/g. The LOQs were evaluated by determining the lowest concentration spike for samples where accuracy and repeatability were satisfactory (within 70–120% and less than 20%, respectively). The estimation of LOD was calculated from the variability of the blank

signals read from the calibration curve. The standard deviation (SD) of the blank amount was used for determining the LODs, i.e. mean plus 3xSD. The single analytical LOD of all terpenoids was selected to facilitate the method application in routine work and should be above all LODs calculated. Generally, LODs were estimated as one-third or half of the LOQs.

The selectivity of compounds for identification and confirmation of 21 terpenes when samples were analyzed, in all cases, should comply with three criteria. Firstly, same retention time as the standard (±0.1 min); secondly, for GC-MS/MS, 2 product ions analyte peaks in the extracted ion chromatograms must fully overlap; and finally, ion ratios from sample extracts should be relatively within ±30% of the average of calibration standards from the same sequence.

Results

Under the optimized chromatographic gradient temperature, all 21 mono- and sesquiterpenoids of interest were separately resolved using the selected reaction monitoring (SRM) mode (Table 1). The results from method validation showed that the method was specific and selective. The method blank is a complete analysis conducted using the solvents and reagents only, in the absence of any sample, and also known as a reagent blank or procedural blank with no interference in the chromatography system. The analytical peaks obtained by the injection of the cannabis leave sample blank, extract of a sample known not to contain detectable levels of the terpenes sought, aliquot into GC-MS/MS with SRM technique shown in Figure 2 presented no interference nor peak at or close to the retention time (RT) of the standard of interest. Moreover, the method was sensitive because the detected terpene peak has a signal-to-noise ratio (S/N) of more than 3 at the limit of detection. The specific ion intensity ratio of each component could improve the degree of confidence in the identification process. This GC-MS/MS SRM analysis allowed the detection and quantification of terpenes and terpenoids in cannabis plant tissues. The total ion chromatogram (TIC) of full chromatograms of 21 terpenes as well as some representative peak shapes of quantification and confirmation transitions is shown in Figure 3. The figure shows four examples of the chromatogram including one quantitation peak and two confirmation peaks at the same retention time in ion overlay display.

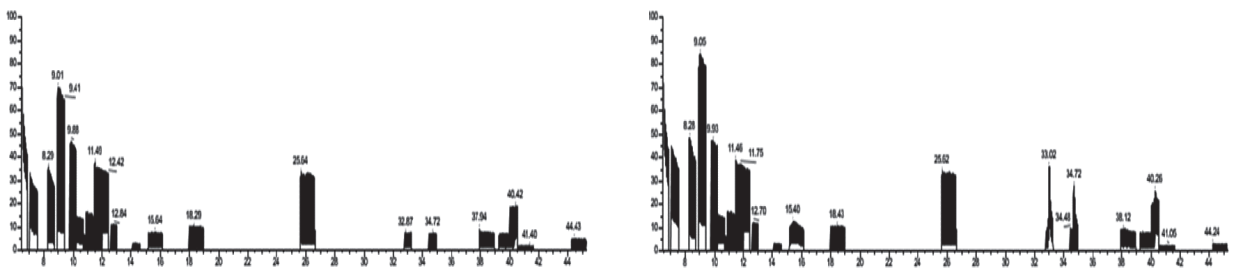

Figure 2 The total ion chromatogram (TIC) of the method blank and sample blank in GC-MS/MS under selected reaction monitoring (SRM) mode

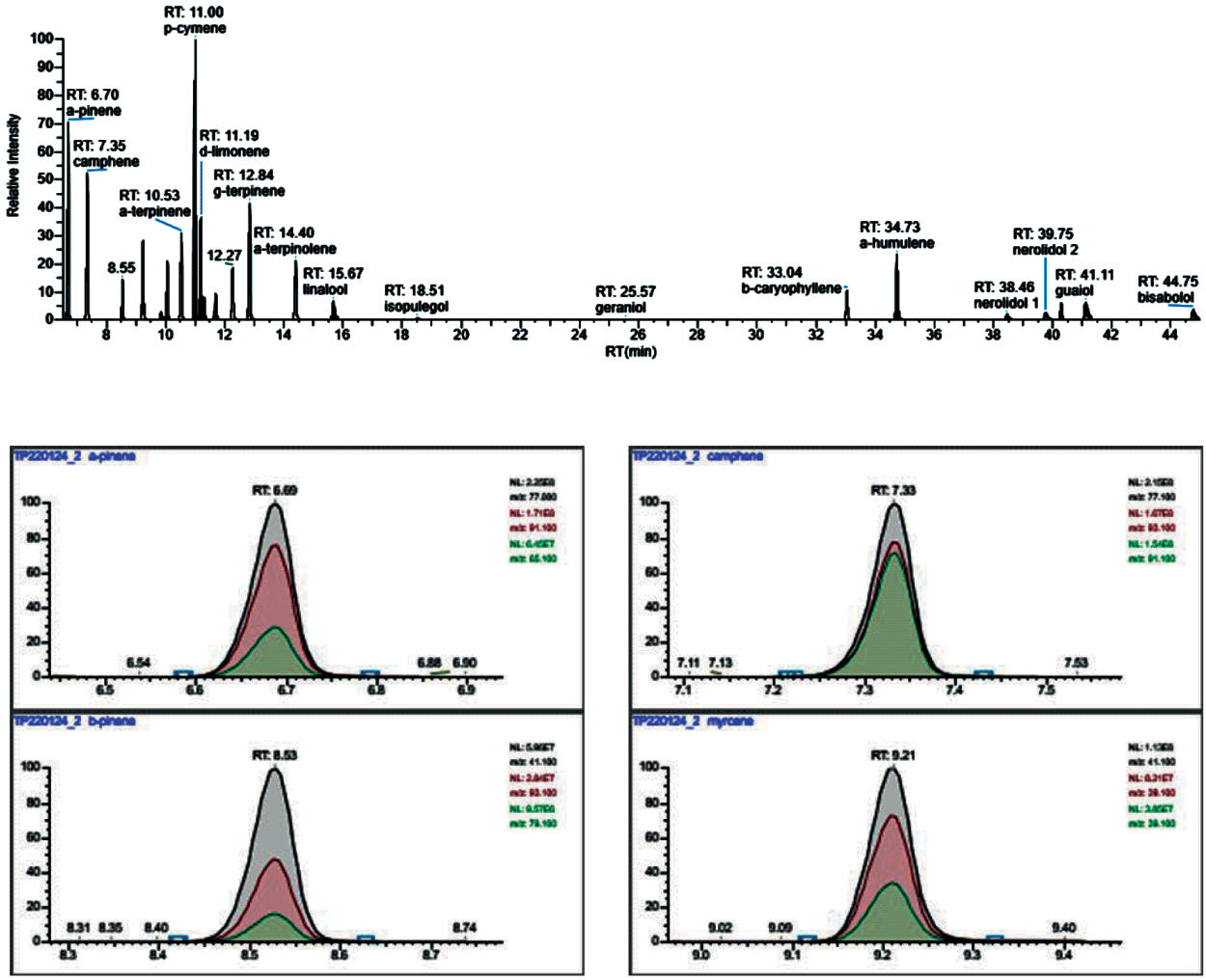

Figure 3 The total ion chromatogram (TIC) of 21 terpenes and some examples of quantification and qualification peaks; α-pipene, camphene, β-pipene and mycene at LOD of 0.05 mg/g

The linearity of calibration curve was assessed and could be obtained by the relationship between the concentration of compounds and respective peak areas (responses). The standard curves with linearity in the range of 0.05 to 100 μg/mL are shown in Figure 4. These curves for 20 substances had the coefficients of determination (r^2) ranging between 0.990 and 0.999, except geraniol (0.973), which was higher than the acceptable value of 0.99.

The limit of detection (LOD) was evaluated as the lowest concentration that could be statistically distinguished from an instrument blank, following the criteria that the signal-to-noise ratio must be greater than 3. The limit of quantification (LOQ) for each terpene was the lowest concentration at which the identification, accuracy, and precision criteria were met, according to the AOAC guidance. The LOD and LOQ for each individual terpene were determined to be 0.03 and 0.05 mg/g in the plant sample, respectively.

The method accuracy and the method precision were determined by spiking a cannabis leave sample with three different concentration levels at LOQ, 10×LOQ and 100×LOQ of

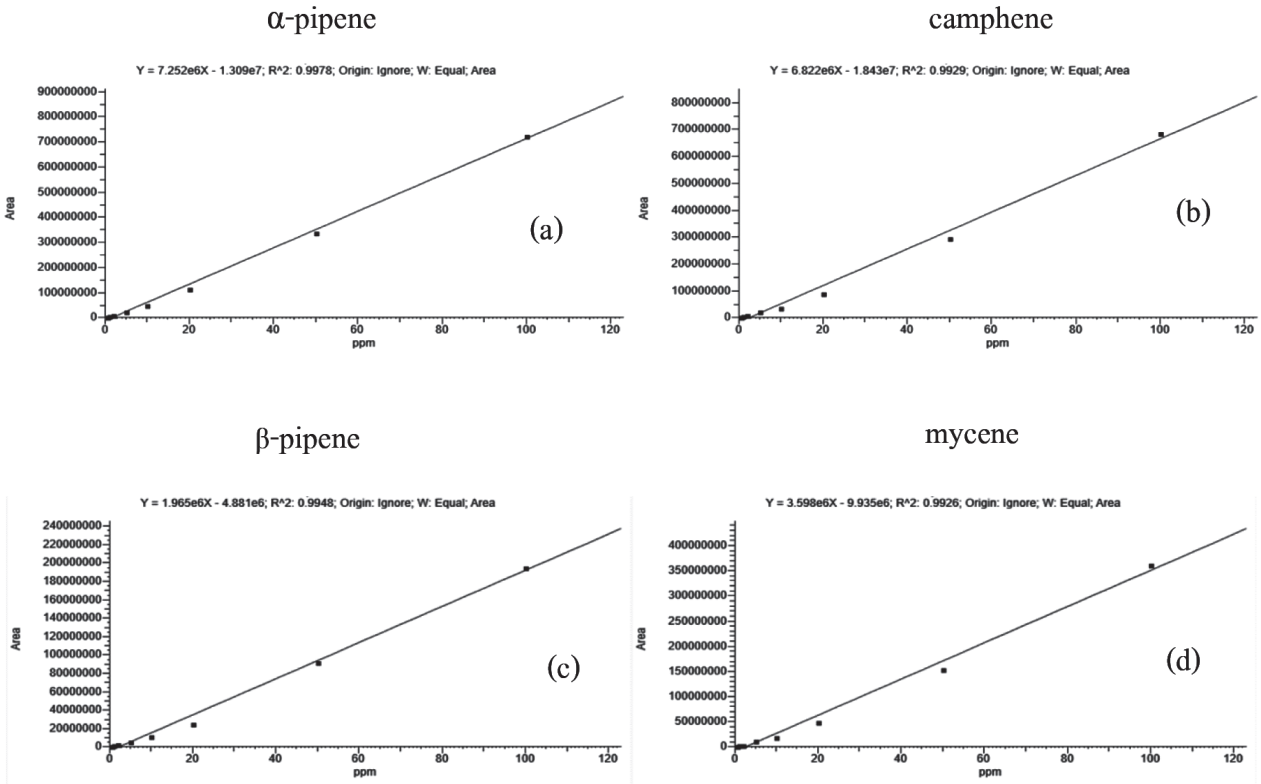

Figure 4 Calibration curves of some terpenes (0.05-100 μg/mL)
a) α -pinene, b) camphene, c) β-pinene and d) myrcene

the standard mixes. The three levels were within the dynamic range of concentrations of all the analytes. Accuracy was determined as the percent recovery from the comparison between the mean concentration obtained and the expected concentration. The precision of the whole method was assessed by calculating the %relative standard deviations (%RSD) for repeatability. Accuracy and precision criteria are listed in Table 2.

Table 2 Acceptable criteria for analyte recovery and HorRat at different concentrations according to AOAC 2016[18]

Analyte concentration	%Recovery	HorRat
10 ppm	80–110	0.3 ≤ HorRat ≤ 1.3
100 ppm	90–107	0.3 ≤ HorRat ≤ 1.3
> 0.1%	95–105	0.3 ≤ HorRat ≤ 1.3

The method accuracy was determined by spiking a cannabis blank sample with three different concentration levels (LOQ, 10×LOQ and 100×LOQ) of the standard mixes as presented in Figure 1. All the tested terpenoids showed accuracies (%recovery) within 92.3–105.1%. The precision was calculated as repeatability represented by %RSD and Horwitz ratio (HorRat). The Horwitz ratio shown in Table 2 is the ratio of the observed RSD(r) from

the experiment and the corresponding predicted RSD(R) calculated from the Horwitz equation. The data showed the precision parameters were between 1.6% and 13.2% for %RSD and ranging from 0.3 to 1.1 for HorRat. The working range was estimated by spiking triplicates of five different concentrations (0.05, 0.1, 0.5, 1, and 5 mg/g). For most of the analytes studied, the linearity within the working range ($r^2 > 0.99$) was found to be 0.05–5 mg/g in the sample. The validation parameters for the GC-MS/MS method including linearity, accuracy and precision are shown in Table 3.

According to the acceptable criteria for analyte recovery and HorRat at different concentrations proposed by AOAC 2016 in Table 2, the method was accurate and fit for the intended use of the quantification procedure. Overall, these results indicated the good efficiency of the extraction and instrumental protocol. Nevertheless, the accuracy of the whole method depends on the natural variability of terpenoids in the inflorescences and the precision of preparations (spiking a small volume of standards onto 0.1 g sample).

The results of the measurement uncertainty were estimated according to the ISO GUM approach or bottom-up approach (at a confidence level of approximately 95%, k = 2). An example of the estimation of measurement uncertainty value of d-limonene in dried cannabis leaves was shown in Figure 5. The major contributing factors were sample weight (42%), method precision (27%) and standard concentration (21%), while other elements such as bias or recovery (5%) and calibration curve (C_0: 3%) contributed minimally as shown in Figure 5.

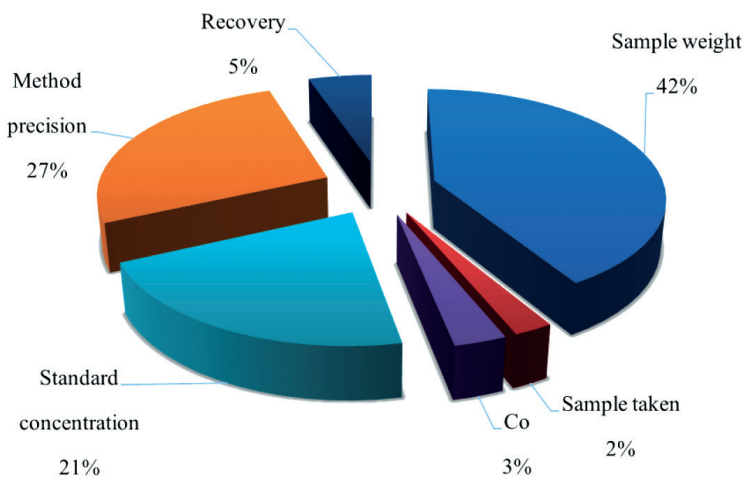

Figure 5 Contribution of the different sources to the overall combined uncertainties of d-limonene in dried cannabis leaves

Table 3 Validation parameters for the GC-MS/MS method: linearity, accuracy and precision (n = 10)

Compounds	Linearity (r^2)	Spiked-1 (0.05 mg/g)				Spiked-2 (0.5 mg/g)				Spiked-3 (5 mg/g)			
		Accuracy		Precision		Accuracy		Precision		Accuracy		Precision	
		Mean	%Rec	%RSD	HorRat	Mean	%Rec	%RSD	HorRat	Mean	%Rec	%RSD	HorRat
3-carene	0.9917	0.0475	95.0	2.7	0.3	0.4904	98.1	5.8	0.4	4.9524	99.0	3.5	0.3
α-humulene	0.9930	0.0466	93.2	11.0	0.9	0.4920	98.4	3.5	0.3	5.0214	100.4	3.3	0.3
α-ocimene	0.9929	0.0507	101.4	5.3	0.4	0.4915	98.3	4.2	0.3	4.9537	99.1	4.6	0.4
α-pinene	0.9978	0.0472	94.3	7.1	0.6	0.4718	94.4	4.1	0.3	5.0989	102.0	3.8	0.3
α-terpinene	0.9907	0.0511	102.1	6.6	0.5	0.4885	97.7	6.9	0.5	4.9116	98.2	4.0	0.3
α-terpinolene	0.9906	0.0508	101.5	6.8	0.5	0.4730	94.6	4.6	0.4	5.0248	100.5	4.7	0.4
β-caryophyllene	0.9928	0.0521	104.2	3.0	0.3	0.4764	95.3	2.7	0.2	4.9561	99.1	2.3	0.3
bisabolol	0.9974	0.0461	92.3	7.8	0.6	0.4810	96.2	4.8	0.4	5.0407	100.8	3.7	0.3
β-ocimene	0.9928	0.0462	92.5	11.3	0.9	0.4968	99.4	4.6	0.4	5.0001	100.0	3.7	0.3
β-pinene	0.9948	0.0491	98.1	6.6	0.5	0.4861	97.2	4.5	0.4	4.7611	95.2	3.2	0.3
camphene	0.9929	0.0492	98.3	3.7	0.3	0.4925	98.5	5.5	0.4	5.1031	102.1	3.4	0.2
caryophyllene oxide	0.9934	0.0486	97.3	9.9	0.8	0.4796	95.9	4.9	0.4	5.1529	103.1	4.4	0.3
cineole	0.9932	0.0494	98.7	5.0	0.4	0.4953	99.1	5.3	0.4	5.1147	102.3	2.4	0.3
d-limonene	0.9990	0.0517	103.5	4.3	0.3	0.4961	99.2	3.3	0.3	5.0936	101.9	3.0	0.3
geraniol	0.9986	0.0513	102.6	5.3	0.7	0.4963	99.3	3.3	0.3	4.1202	82.4	5.1	0.5
γ-terpinene	0.9939	0.0502	100.4	7.8	0.6	0.4975	99.5	4.4	0.3	4.9158	98.3	9.7	0.8
guaiol	0.9734	0.0463	92.5	8.1	0.7	0.4836	96.7	4.4	0.3	5.1950	103.9	6.4	0.5
isopulegol	0.9948	0.0464	92.8	13.2	1.1	0.4883	97.7	5.2	0.4	5.1248	102.5	3.6	0.3
linalool	0.9900	0.0495	98.9	5.9	0.4	0.4954	99.1	4.5	0.3	5.0463	100.9	3.1	0.3
myrcene	0.9926	0.0510	102.0	6.2	0.5	0.5017	100.3	5.0	0.4	5.0670	101.3	3.4	0.3
nerolidol 1	0.9969	0.0487	97.4	10.2	0.8	0.5145	102.9	4.4	0.3	5.1062	102.1	1.6	0.3
nerolidol 2	0.9923	0.0474	94.9	9.5	0.8	0.4848	97.0	4.6	0.4	5.1425	102.9	4.7	0.3
p-cymene	0.9914	0.0526	105.1	2.7	0.3	0.4977	99.5	4.8	0.4	5.0283	100.6	3.4	0.3

The developed GG-MS/MS method was applied to the determination of terpene profile characteristics of GG#1 cannabis variety grown in our Department. Four parts of the plant including inflorescences, leaves, stems and roots were selected to be analyzed. The blind selection of 20 individual plants was harvested from a cannabis field trial plantation of approximately 100 plants from the same seed species. A total of 80 samples were tested for the quantification of terpenes to give the unique profile for the further species registration.

A variation in the total and individual terpene content between four different parts of the plant was observed. Table 4 shows, in general, that the concentration of all terpenes in inflorescence was higher than in other parts of the plant. For example, the content of α-pinene was around 10 times higher in the flowers than in leaves and stems. Three compounds, which were caryophyllene oxide, geraniol, and isopulegol, were not traceable in any samples. Leaves and stems presented slightly the same content level of terpenes while roots did not contain any terpene in plant tissue. Figure 6 shows the distribution of cannabis terpenes present in four parts of GG#1 cannabis variety. These observable data may help in the selection of a specific variety or to differentiate one variety from another on the basis of terpene content profile. In our works, as all plants were grown in a specific area and under the same condition, the consistency in the terpene concentration was consequently noticed in these materials. More studies should be undertaken using the principal component analysis to investigate the distribution of terpenes in the same cannabis strain produced in different areas and with changes in climates, resources, and farmers' cultivation practices.

Table 4 Quantitative analysis of four parts of cannabis plant samples in mg/g (n = 20)

Terpenes	Average concentration ± SD (mg/g)			
	Inflorescences	Leaves	Stems	Roots
3-carene	0.83±0.02	0.06±0.01	0.11±0.01	ND
α-humulene	3.54±0.14	0.18±0.01	0.13±0.01	ND
α-ocimene	1.29±0.02	0.11±0.01	0.15±0.01	< LOQ
α-pinene	1.54±0.09	0.09±0.02	0.13±0.01	ND
α-terpinene	2.54±0.03	0.22±0.01	0.20±0.01	< LOQ
α-terpinolene	2.15±0.06	0.21±0.01	0.16±0.01	< LOQ
β-caryophyllene	5.25±0.27	0.17±0.02	0.12±0.01	ND
bisabolol	2.52±0.06	0.14±0.01	0.17±0.01	ND
β-ocimene	1.43±0.02	0.11±0.01	0.16±0.01	< LOQ
β-pinene	0.47±0.06	< LOQ	ND	ND
camphene	0.86±0.01	0.06±0.01	0.10±0.01	ND
caryophyllene oxide	ND	ND	ND	< LOQ
cineole	0.37±0.03	0.05±0.01	0.06±0.01	ND
d-limonene	0.95±0.03	< LOQ	0.11±0.01	ND
geraniol	ND	ND	ND	ND
γ-terpinene	0.76±0.01	0.09±0.01	0.09±0.01	ND
Guaiol	2.82±0.08	0.17±0.01	0.19±0.01	ND
isopulegol	ND	ND	ND	ND
linalool	1.12±0.02	ND	0.09±0.01	< LOQ
myrcene	1.03±0.02	< LOQ	0.12±0.01	ND
nerolidol 1	1.5±0.05	ND	0.09±0.01	ND
nerolidol 2	2.57±0.06	0.07±0.01	0.19±0.01	ND
p-cymene	0.54±0.04	0.08±0.01	0.09±0.01	ND

ND = not detected; < LOQ = less than 0.05 mg/g

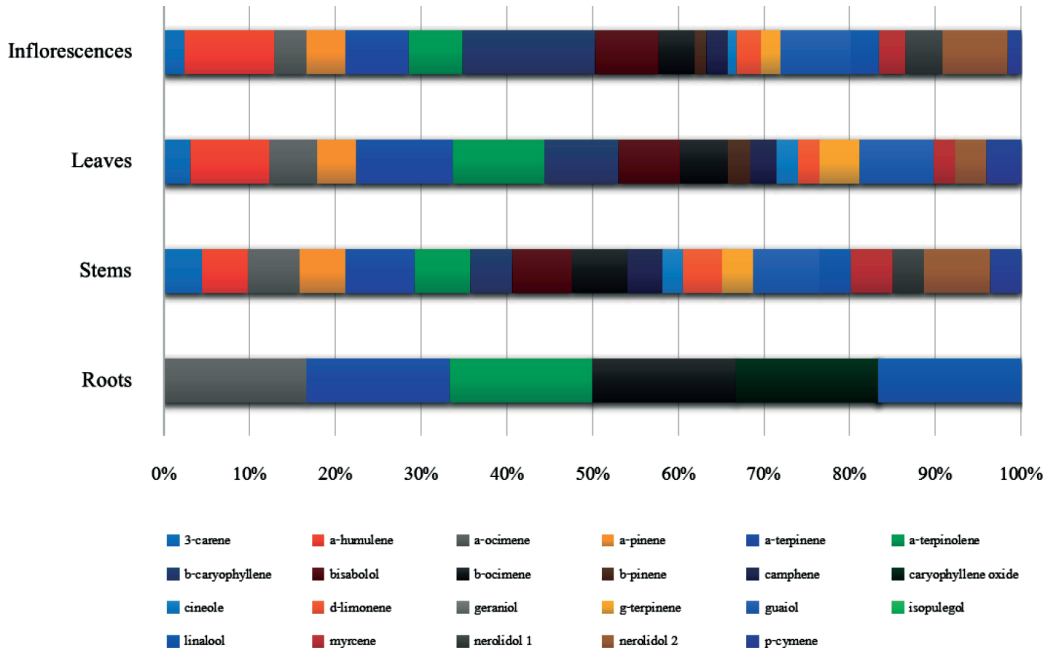

Figure 6 Distribution of cannabis terpenes present in four parts including inflorescences, leaves, stems and roots of GG#1 cannabis variety

Discussion

The challenge for the analysis of different classes of properties of terpenes is the selection of solvent of extraction. Even though the optimum solvent composition for terpene and cannabinoid compounds differs depending on the mixture of analytes of interest,[19] in this work we found that ethyl acetate is the solvent of choice and represents an excellent extraction property with a compromised recovery rate obtained. The use of small amounts of organic solvent results in low environmental impact with less waste to be managed.[20] Another important parameter highly affecting the results was the sample concentration before instrument injection. Due to the powerful instrument sensitivity given by GC-MS/MS with the aid of triple quadrupole technique, the sample concentration of 0.005 g/mL was prepared and was enough for clear separate detection. The terpene testing still provides sufficient concentrations of terpenes in working sample solutions to be quantified without concentrating them. Because the terpenes are very volatile and significantly lost during evaporation step, our technique has been shown to be suitable for recovery reservation. To identify individual terpene by gas chromatography using analytical column, the analytes were separated in a fused silica capillary column DB-5MS with ubiquitous stationary phase based on 5% phenyl/95% dimethylpolysiloxane. This middle polar column was selected firstly because it was available in our laboratory as a column of choice in pesticide residue analysis. The terpenoid compounds were therefore easily separated between each other using the temperature gradient programme[21] set by constant low rate of temperature changes due to a big difference in volatility of the compounds.

The developed method presents a great analytical performance within a reasonable analysis timeframe, since it provides separation of multiple components in cannabis plant. Twenty-one analytes were separated with excellent resolution and the baseline was clean enough for identification and confirmation. Validation data including the linearity, accuracy and precision parameters shown in Table 3 confirmed the suitability of the method also for quantitative use. Sufficiently low sensitivity limits given by the instrument set-up were important especially from the terpene standpoint, and on the other hand, accurate results during extraction and analytical step allowed work with non-concentrated or non-diluted working sample solutions. This is a great advantage especially in view of the process simplicity.

The developed extraction and instrumental analyses provide sufficient sensitivity to identify target terpenes highly present in cannabis plants. Figure 3 shows the separation of 21 compounds and isomers; and in Table 4, the identification of terpenes with ion intensity ratio used for confirmation purpose is provided. Eighteen terpenes were abundantly present in inflorescence samples and could be used as complementary chemical markers in the qualification of Cannabis genus plants. A variation in the total and individual terpene content between parts of plant was observed. Within the inflorescences from cannabis flower, the content of terpenes was generally higher in the other parts. For example, the content of α-humulene was around twenty times higher in the leaves and stems (0.18 and 0.13 mg/g), while the compound was not detected in the roots. The trend was observed continually in the rest of target terpenes. These observations may help in the selection of a specific variety or to differentiate one variety from another on the basis of terpene content. More studies are underway using principal component analysis to investigate the distribution of terpenes among different Thai cannabis varieties produced in Thailand under medical cannabis laws.

Conclusion

In this study, a GC-MS/MS method was developed for the simultaneous analysis and quantification of 21 terpenes. Using tandem mass spectrometer offers the added benefit of spectral confirmation of peaks to ensure that identification is accurate with no co-eluting interferences. The method was shown for cannabis terpenoids; however, it could also be used for other natural terpenes as well. The extraction and instrument method were fully validated showing the reliability and practicability of the method. A single solvent was used to demonstrate an environmentally friendly and simple microextraction of hemp and cannabis plant materials. The validation parameters, including linearity, sensitivity, selectivity, precision, and accuracy, were acceptable within analytical criteria. The working range covered the whole concentration of terpenoids present in GG#1 cannabis strain. This method has been successfully applied to the analysis of cannabis inflorescences, leaves, stems and roots. Data of terpenes profile from this work, which will be incorporated with botanic, genetic, and other chemical information from our Department along with the medical efficacy and safety of cannabis varieties, will be registered by the authority for the official recognition in Thailand.

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การพัฒนาและทดสอบความใช้ได้ของวิธีตรวจวิเคราะห์สารเทอร์พีนและเทอร์พีนอยด์ในกัญชากัญชง โดยเทคนิค GC-MS/MS

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บทคัดย่อ เทอร์พีน (Terpenes) เป็นหนึ่งในองค์ประกอบหลักที่มีอยู่ในพืชกัญชากัญชง ซึ่งผลของการรักษาอาการผิดปกติของร่างกายและสมองโดยใช้กัญชากัญชงขึ้นอยู่กับองค์ประกอบและสัดส่วนจำเพาะของเทอร์พีน การบำบัดความผิดปกติทางอารมณ์และโรควิตกกังวลด้วยกัญชากัญชงต้องอาศัยการทำงานแบบ entourage effect ของสารกลุ่มเทอร์พีนร่วมกับสารกลุ่มแคนนาบินอยด์ การกำหนดปริมาณและสัดส่วนของเทอร์พีนในกัญชากัญชงจึงมีความสำคัญต่อการคัดเลือกพืชสมุนไพร การศึกษานี้ได้พัฒนาวิธีวิเคราะห์โดยใช้เครื่อง triple quadrupole mass spectrometer โดยเทคนิค SRM และตรวจสอบความใช้ได้ของวิธีเพื่อหาชนิดและปริมาณของเทอร์พีนรวม 21 ชนิด เตรียมตัวอย่างโดยการสกัดส่วนของพืชด้วยเอทิลอะซีเตตตามด้วยการแยกสารด้วยการสั่นสะเทือนจากคลื่นอัลตราโซนิคและหมุนเหวี่ยงให้ตกตะกอน สารสกัดตัวอย่างที่ได้ถูกฉีดโดยตรงเข้าเครื่องมือวิเคราะห์ ผลการทดสอบพบช่วงความเป็นเส้นตรงอยู่ระหว่าง 0.05–5 มิลลิกรัมต่อกรัม โดยมีค่า $r^2 > 0.99$ ความแม่นจากค่าการคืนกลับเฉลี่ยของเทอร์พีนทุกชนิดยกเว้น geraniol ในตัวอย่างมีค่าอยู่ระหว่าง 92.3–105.1% ความเที่ยงของวิธีแสดงด้วยส่วนเบี่ยงเบนมาตรฐานสัมพัทธ์ (%RSD) อยู่ในเกณฑ์ยอมรับระหว่างตั้งแต่ 1.6–13.2% ขีดจำกัดของการตรวจพบและขีดจำกัดของการตรวจวัดเชิงปริมาณของเทอร์พีนมีค่าเป็น 0.03 และ 0.05 มิลลิกรัมต่อกรัม ตามลำดับ วิธีนี้เป็นวิธีที่มีความจำเพาะเจาะจง เชื่อถือได้ แม่นยำ และสามารถนำไปใช้กับการหาปริมาณเทอร์พีนที่เป็นองค์ประกอบในส่วนต่าง ๆ ของกัญชากัญชงที่พัฒนาสายพันธุ์โดยกรมวิทยาศาสตร์การแพทย์ ได้แก่ ช่อดอก ใบ ต้น และราก เพื่อประกอบการขึ้นทะเบียนพันธุ์พืชกับกรมวิชาการเกษตรต่อไป

คำสำคัญ: เทอร์พีน, เทอร์พีนนอยด์, กัญชา, กัญชง, GC-MS/MS