

Development and validation of HPLC and GC methods for quantification of cannabinoids and terpenes extracted by ultrasound assisted extraction technique

Masoumeh Lazarjani^a, Ali Seyfoddin^{ib*}, Thao T. Le^{ib}, Tony Chen^a

^a Department of Science, Faculty of Health and Environmental Science, Auckland University of Technology, Auckland, New Zealand.

*Corresponding author: Ali.seyfoddin@aut.ac.nz

Cannabis exhibits a wide array of therapeutic effects due to its diverse and potent compounds. High-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) are the primary techniques used to quantify and qualify cannabinoids and terpenes in cannabis. This paper rigorously validates HPLC and GC-MS methods for quantifying cannabinoids and terpenes in cannabis plants. Cannabinoids and terpenes were extracted using an optimised ultrasound assisted extraction (UAE) method. The HPLC and GC-MS methods were validated by assessing linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, precision (inter-day and intra-day), accuracy (inter-day and intra-day), and matrix effect for 12 cannabinoids and 30 terpenes using appropriate standards. The HPLC and GC results fell within acceptable ranges (for HPLC, recovery between 73-121%, precision, accuracy and matrix effect all were below 10%; and for GC, recovery between 79-91%, precision, accuracy and matrix effect all were below 10%) demonstrating the accuracy and reliability of the validated method. The findings of this study can be used effectively to measure these important compounds in cannabis samples, facilitating quality control and standardisation of cannabis-based products for therapeutic applications.

Keywords: HPLC analysis; gas chromatography; cannabinoids; terpenes; quantification.

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Introduction

The cannabis plant is a complex mixture comprising more than 1000 different compounds, including 170 cannabinoids and over 250 terpenes [1, 2]. Cannabis has been used for its therapeutic properties for thousands of years [3]. The therapeutic properties of cannabis are attributed to cannabinoids [4]. Cannabinoids, notably tetrahydrocannabinol (THC) and cannabidiol (CBD), are primarily found in the resin produced by the trichomes are mostly concentrated in female flowers [3, 5]. A conventional classification model of cannabinoids is due to their chemical contents dividing them to subclasses including cannabigerol (CBG), THC, CBD, cannabichromene (CBC), cannabinol (CBN), (-)- Δ^8 -trans-tetrahydrocannabinol (Δ^8 -THC), cannabicyclol (CBL), cannabinodiol (CBND), cannabielsoin (CBE), cannabitrilol (CBT) and miscellaneous [6]. Numerous studies have highlighted the therapeutic properties of cannabinoids, including their analgesic, anti-inflammatory, and neuroprotective effects. On the other hand, terpenes synthesised in cannabis trichomes are produced to attract pollinators, repel predators, and protect the plant from environmental stressors [7]. Myrcene, a prominent cannabis terpene, is linked to sedative and muscle relaxant properties, along with potential anti-inflammatory and analgesic effects [7]. Limonene has been reported to have uplifting and mood-enhancing effects, along with potential anti-anxiety and antidepressant properties [8]. Various methods exist for extracting cannabinoids and terpenes from medicinal cannabis. Lewis-Bakker (2019)

highlights solvent extractions, supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE) as common methods[9]. De Vita *et al.* (2020) demonstrated that UAE provides the highest yield compared to other methods. Unlike SFE, UAE isn't limited by specific temperature and pressure requirements and extracts a broader range of compounds. It's also faster than Soxhlet and preserves all terpenes, unlike MAE, which may lead to terpene loss due to high temperatures. Given these advantages, UAE was selected for sample preparation in this study [10].

A variety of analytical techniques have been developed for quantification and qualification of cannabinoids, terpenes, and other compounds in cannabis plant. The aim of this study was to investigate various quantification techniques for cannabinoids and terpenes in cannabis, to identify the most suitable method among those described in the existing literature. Furthermore, it is aimed to present the validation of the selected method to ensure its reliability and accuracy. By thoroughly evaluating different approaches and conducting method validation, this research contributes to the advancement of analytical techniques for the precise quantification of cannabinoids and terpenes in cannabis [11, 12].

There are several quantification methods that can be applied for determination of cannabinoids and terpenes [13]. However, HPLC and GC-MS offer distinct advantages such as sensitivity and accuracy and are frequently chosen due to their effectiveness in separating and detecting these compounds [14].

HPLC and GC method validation

Method validation is crucial for ensuring the reliability, accuracy, and consistency of analytical procedures before their routine application [15]. Selectivity and specificity, linearity, accuracy, precision, LOD and LOQ are the most critical parameters to be optimised in method validation for both HPLC and GC, according to AOAC, FDA, ICH, and USP guidelines.

Selectivity and specificity: selectivity refers to the ability of the method to separate different analytes from each other. Specificity, refers to the ability of the method to accurately identify and quantify a particular analyte in the presence of other components [16]. To measure selectivity, chromatographic blanks from sample containing no interest compound will be evaluated in the expected timeframes of the interest compound peak [17]. In another word, selectivity of cannabinoids can be detected by measuring retention times [18]. While, based on Ibrahim *et al.* (2019) selectivity of terpenes is assessed by the resolution of the peaks in the GC chromatogram, which must be less than two. Resolution value of ≥ 2 is often considered as a minimum requirement for adequate separation between peaks. This value ensures that there is a significant gap between adjacent peaks, allowing for reliable identification and quantification of individual components [19].

Linearity: Linearity refers to the relationship between the observed response and the concentration of the interest compound in the sample [18]. Linearity is measured by introducing a range of stock solution of standards or diluted stock solution of standards into the mobile phase at a minimum of five various concentrations [17]. It is a common procedure to evaluate the correlation coefficient (r) to verify that a calibration curve is linear. A correlation coefficient close to one ($r = 1$) is regarded adequate proof that the linearity of experiments is correct [20].

Accuracy: The degree to which the test results and the standard reference value align is referred to as accuracy [18]. Araujo (2009) mentioned accuracy as the most important parameter in method validation which involves assessing the impact of systematic errors on a specific method. In the same study, four approaches have been demonstrated for measuring accuracy including sample analysis by using a specific reference material and comparing the results with standards, spiking a defined concentration of sample into a blank matrix and calculating the percentage of recovery, using reference methods as comparison with the results of an unknown sample, and using the internal standard technique to determine the analytical concentration in the sample [20]. The ICH suggests measuring the accuracy by conducting a minimum of nine data points across three concentration levels (low, medium, and high) that correspond to the entire analytical range studied (3 concentration levels, 3 replicates per level = 9 data points) (ICH, 1996).

Precision: When a method is repeated on multiple samples, the level of consistency among individual test results is called precision. Precision is determined by injecting a series of standards or analysing numerous samples from a

uniform batch. Precision is also called relative standard deviation (%*rsd*) which can be calculated by Formula 1 with the use of standards deviation (SD) and the Mean values [17]:

$$\text{Formula (1): } \%RSD = \frac{SD}{Mean} \times 100$$

Limit of detection (LOD) and limit of quantification (LOQ): The lowest concentration at which the device can detect but not quantify is referred to as LOD. LOQ, on the other hand, is the lowest concentration that the device can both detect and quantify [17]. Araujo (2009) presented a scheme containing the main approach to calculate LOD based on the literature. In the mentioned study LOD can be determined in three ways including visually by spiking samples with known concentration of interest compound and assessing the level at which the interest compound can be identified. Another way is to determine the signal to noise relationship by comparing analytical signals at known low concentrations with blank sample signals up to an analytical concentration that provides a signal comparable to three or two times the blank sample's standard deviation. The quotient of standards deviation and the slope of regression curve in the final approach for LOD calculation [20]. Similarly, Rozet *et al.* (2007) introduces several LOQ calculation methods, including visual evaluation, signal to noise ratio, and standards deviation to slope ratio, with the latter being the most common [21]. The aim of this study is to validate a novel and reliable HPLC and GC methods for the analysis of cannabinoids and terpenes, using an optimised ultrasound-assisted extraction technique.

Experimental section

Chemicals and standards

HPLC grade methanol, ethanol, and hexane was purchased from Sigma Aldrich, New Zealand. %100 formic acid (FA) and Acetonitrile were purchased from Thermo Fisher Scientific, New Zealand. Helium gas (BOC), dichloromethane and methanol were purchased from Thermo fisher Scientific, New Zealand. A mixture of cannabinoids including Δ^8 -THC, Δ^9 -THC, CBN, CBG, CBD, CBC, CBDV and THCV with 500 $\mu\text{g/mL}$ of each analyte in methanol as well as four individual standards of acidic forms of CBD, THC, CBG and CBL with 1 mg/mL concentration in acetonitrile were purchased from Cerilliant Corporation (USA). Terpenes standards were purchased from Merck, New Zealand in two mixtures including 30 terpenes with 2000 $\mu\text{g/mL}$ of each analyte in methanol.

Instrumentation conditions

A probe UP100H ultrasound device from Hielcher, Germany is used for the entire extraction process. The centrifugation is performed by a 158OR Gyrozen, South Korea. HPLC-UV analysis used a Shimadzu LC-20 series HPLC with an Agilent Zobrax Eclipse Pluc C-18 column, achieving separation in 26 min with an isocratic method of

0.1% formic acid in MilliQ Water (16%) and acetonitrile (84%). Detection was at 280 nm, with a 5 μ L injection volume. GC analysis utilized an Agilent 7890A GC with a 5977A MSD, using an Agilent DB-5ms UI column. The inlet temperature was 250 °C, with a 1 μ L sample injected in split mode (10:1). The initial column temperature was 60 °C for 4 min, then ramped at 8 °C/min to 120 °C for 5 min, further ramped at 8 °C/min to 200 °C, and finally ramped at 20 °C/min to 310 °C, held for 3 min. MSD settings included a transfer line at 250 °C, ion source at 250 °C, and quad at 150 °C.

Sample preparation

One chemovar of cannabis plant was kindly provided by Helius Therapeutics Ltd for this study. A fine cannabis powder was prepared using a coffee grinder for about 90 seconds up to an average size of 600 μ m to 1.3 mm based on the SEM photos (Figure 1). UAE was conducted on 1 g of powder in 10 mL ethanol: hexane (1:1, v: v) for 7 minutes using a sonicator with probe number 6 in an ice bath. Extracts were centrifuged at 4000 rpm for 20 minutes and filtered with a 0.45 μ m syringe filter before injection into GC or HPLC, diluted to 10 and 100 folds respectively. A 50 mL stock of 100 μ g/mL biphenyl in HPLC grade methanol served as the internal standard for GC. As it is not possible to obtain cannabis samples without terpenes and cannabinoids, a blank matrix was prepared by sonication of 30 grams of plant material in chloroform and methanol (1:1, v: v) which are stronger solvents than ethanol and hexane to extract as many constitute as possible for 8 hours, followed by filtration and overnight freeze-drying. UAE extraction on the freeze-dried material showed minimal terpene content and residual cannabinoids. To determine spike recovery, residual cannabinoid background concentrations were subtracted from the analysis results. [22].

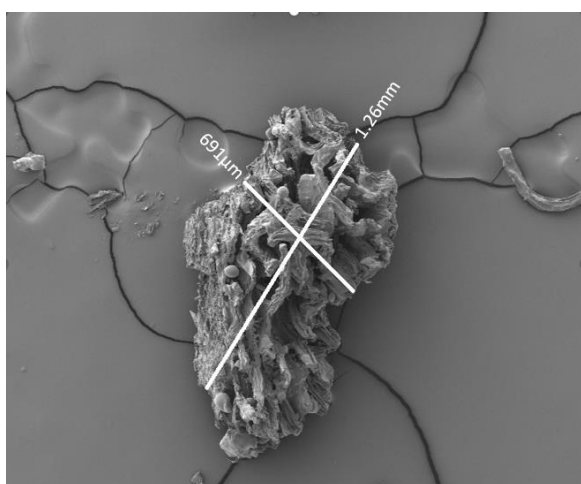


Figure 1. SEM photo of cannabis particle size after 90 seconds grinding.

Method validation

The HPLC and GC methods were validated for linearity, precision, recovery, accuracy, matrix effect, LOD and LOQ.

Linearity

Calibration plots were generated for various concentrations of the target analytes to complete linearity studies [23]. In this case, for HPLC, calibration curves were prepared gravimetrically in diluent solution at concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 μ g/mL from 12 cannabinoids standards. For GC, on the other hand, a series of standards from 40, 20, 10, 4, 2, and 1 μ g/mL were injected to methanol using 2 terpenes mixture and internal standard.

Precision

Intra- day repeatability was calculated using the Formula (2) based on Pugliese *et al.* (2020) and inter- day repeatability was calculated using the Formula (3):

Formula (2): (standard deviation of blank matrix (BM) spike over 6 same-day injections/ mean peak area of blank matrix (BM) spike over 6 same-day injections) $\times 100$.

Formula (3) by [24]: (standard deviation of blank matrix (BM) spike over 6 injections in 6 continuous days injections/ mean peak area of blank matrix (BM) spike over 6 injections in 6 continuous days injections) $\times 100$.

Recovery

Recovery of the analytes were studied by spiking standards into the BM in three concentrations: low, medium, and high levels (1, 10 and 20 μ g/mL for GC), adding solvents and conducting extraction following sample preparation section. The recovery was calculated based on Formula (4):

Formula (4): (Measured amount of analyte after extraction / Spiked amount of analyte) $\times 100$.

Based on the United States Pharmacopeia (USP) general chapter validation of compendial procedures, the recovery should be close to 100%, however the range of 80-120% is also acceptable indicating that the method can effectively extract the analyte from the sample matrix and accurately quantify it [25].

Accuracy

Accuracy or bias was performed in 3 levels of concentration (2, 20 and 50 μ g/mL for HPLC and 1, 10 and 20 μ g/mL for GC). Accuracy was calculated using the following formula: Accuracy (%) = [(Measured Value - True Value) / True Value] $\times 100$ where measured value is concentration of spike sample and true value is expected concentration of analyte of interest. Based on the European Medicines Agency (EMA) accuracy or bias less than 10% is acceptable.

Matrix effect

The calculation of matrix effects was achieved using the following the Formula (5).

Formula (5): $[(\text{mean peak area of post-spiked} - \text{peak area of blank matrix}) / \text{peak area of neat standard}] \times 100$.

The levels of spike concentrations and acceptable range for the results were the same as recovery section [24].

LOD and LOQ

The sensitivity of the method was assessed by determining the limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were calculated by identifying the concentration of a diluted standard solution at which the signal-to-noise ratio was around 3 and 10, respectively [26].

Statistical analysis

The data were analysed using Microsoft office Excel. All the experiments were performed at least in triplicates in three individual experiments.

Results and discussion

HPLC method validation for cannabinoids

A mixture of cannabinoids was run to determine retention time (RT) of each analyte. Figure 2 shows HPLC chromatogram of 12 cannabinoids. All the standards were completely separated in distinct picks within 20 minutes in HPLC machine. The first and last cannabinoids separated are CBDV in about 5 minutes and THCA in 20 minutes, respectively.

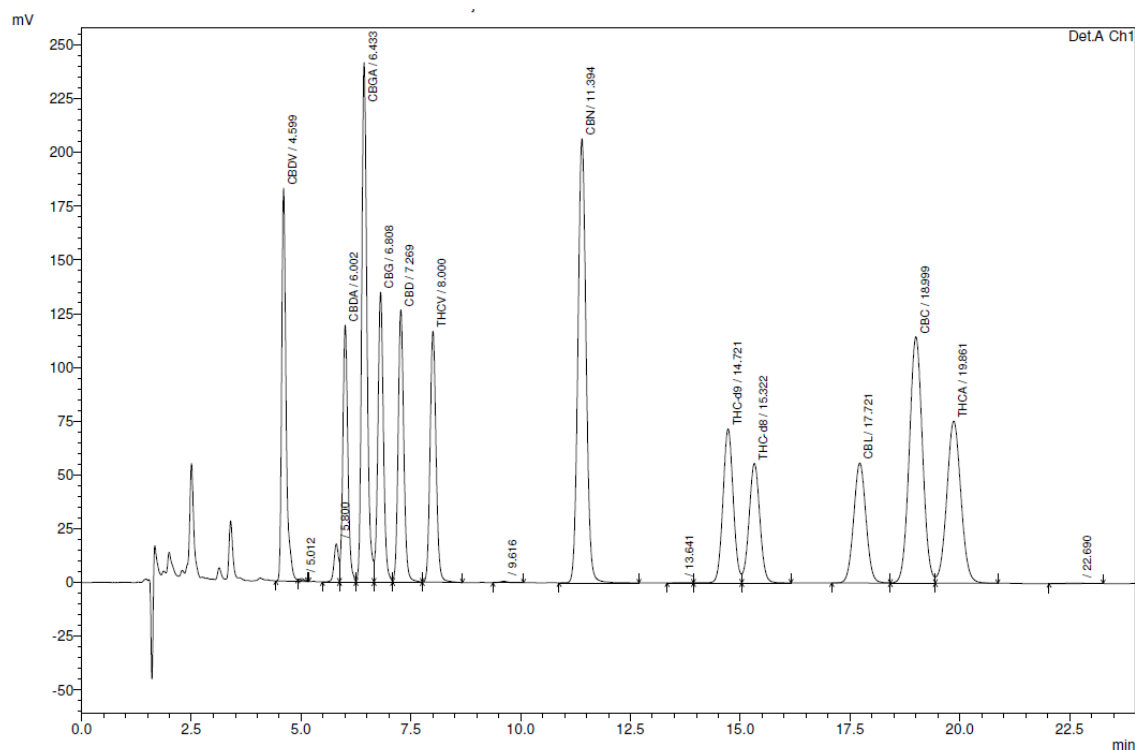


Figure 2. HPLC Chromatogram showing the separation of 12 standards cannabinoids in 20 minutes.

Linearity

Different concentrations of cannabinoids were used to create calibration plots for conducting linearity studies. The calibration curves for the regression were visibly straight and linear (Figure 3). Furthermore, the correlation coefficients of all 12 cannabinoids ranged between 0.9997 to 0.9998 in a 0-100 $\mu\text{g/mL}$ linear range (Table 2). Jin, D., *et al.* (2017) reported the linearity of 10 cannabinoids between 0.9993 to 1.0000 using HPLC [27]. The same results have been shown in De Backer, B., *et al.* (2009) and Patel, B., *et al.* (2017) by using HPLC for 7 and 8 cannabinoids, respectively.

LOD and LOQ

The limit of detection (LOD) and limit of quantification (LOQ) values for cannabinoids were assessed, and the results are presented in Table 1. The LOD values ranged

from 0.2 to 0.45 $\mu\text{g/mL}$, while the LOQ values ranged from 0.6 to 1.5 $\mu\text{g/mL}$. It is important to note that all the LOD and LOQ values obtained for each cannabinoid were lower than the lowest concentration of the standards (3.125 $\mu\text{g/mL}$). These findings indicate that the analytical method employed in this study is highly sensitive, capable of detecting and quantifying cannabinoids at extremely low levels. Brighenti, V. *et al.* (2017) reported LOD values ranging from 0.5 to 0.8 $\mu\text{g/mL}$ and LOQ values ranging from 1.8 to 2.5 $\mu\text{g/mL}$ for various cannabinoids using a similar analytical approach. Similarly, in a recent study by Wu *et al.* (2023), the LOD reported for cannabinoids ranged from 0.01 to 0.5 $\mu\text{g/mL}$ [28]. The preference for HPLC over LCMS-MS in our study stemmed from the abundance of cannabinoids in the samples, rendering HPLC a more suitable and widely adopted instrument within the industry.

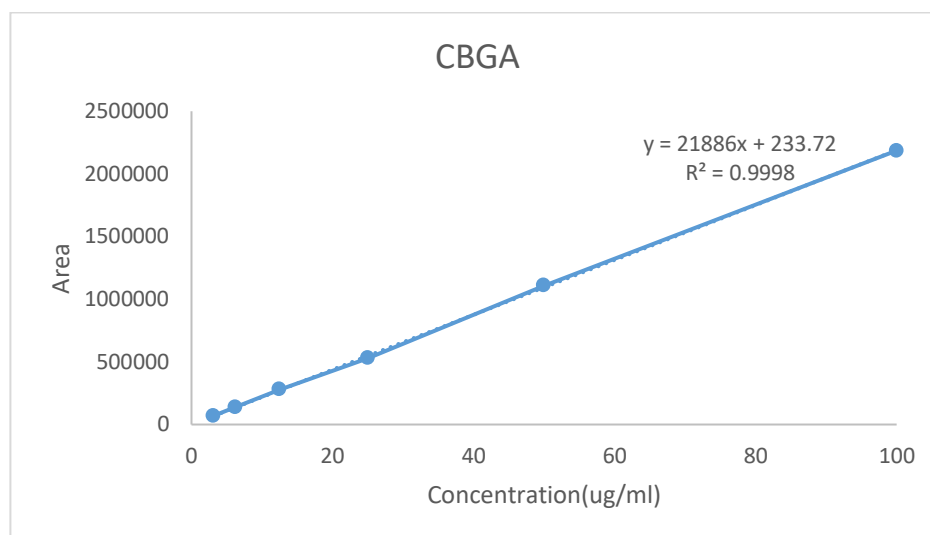


Figure 3. calibration curve of CBGA using HPLC

Table 1. Linearity, LOD and LOQ results for cannabinoids using HPLC (N= 6 replica, 6 times).

Cannabinoids	Coefficient of determination (R^2) (N=6)	LOD ($\mu\text{g/mL}$) (N=6)	LOQ ($\mu\text{g/mL}$) (N=6)
CBDV	0.999	0.335	1.116
CBDA	0.999	0.367	1.226
CBGA	0.999	0.367	1.226
CBD	0.999	0.450	1.502
CBG	0.999	0.367	1.224
THCV	0.999	0.340	1.134
CBN	0.999	0.468	1.560
D9-THC	0.999	0.229	0.766
D8-THC	0.999	0.200	0.669
CBL	0.999	0.351	1.171
CBC	0.999	0.284	0.948
THCA	0.999	0.342	1.140

Recovery

The recovery rates of all cannabinoids analysed in this study exhibited a range of 73-121%. Notably, one sample of Δ^9 -THC demonstrated the lowest recovery rate, approximately 73%, which aligns with the findings reported by Chu and Drummer in 2002 [29]. This can be related to chemical stability and volatility of THC. Δ^9 -THC is less chemically stable compared to other cannabinoids like CBD (cannabidiol). It can degrade into CBN (cannabinol) when exposed to light, heat, or air, leading to lower recoveries during analysis[3]. Conversely, among the samples tested, CBG exhibited the highest recovery rate, reaching approximately 121%. These results are consistent with the findings reported by Debacker *et al.*, who also observed recovery rates above 100% for CBG. Brighenti, V., *et al.* (2017) have shown that the recovery percentages, determined by comparing the results of samples and fortified samples, exceeded 74% and can be regarded as satisfactory [30]. Recovery is a crucial

parameter in analytical chemistry as it assesses the accuracy and reliability of the analysis. A recovery of ≥ 80 would indicate that the method is capable of accurately measuring the concentration of the analyte [22]. In this study, the average recoveries ranging from 80% to 90% suggest that the method performed well in terms of accuracy (Table 2).

Precision

Precision was assessed for each cannabinoid at different concentration levels (2, 20, and 50 $\mu\text{g/mL}$) in both intra-day (6 runs per day) and inter-day (6 runs per day within 6 continuous days) measurements. The precision values for cannabinoids were all below 9.3% for intra-day and 8.7% for inter-day analyses. However, when excluding the results of low concentrations, the precision values decreased to below 3.7% for intra-day and 4% for inter-day measurements (Table 3). These findings demonstrate the high precision and repeatability of the analytical method

employed in this study. In a previous study conducted by De Backer, B., *et al.* (2009), where precision (%RSD) of seven cannabinoids did not exceed 11% [18], the results of this study indicate even lower levels of variability. Patel, B., *et al.* (2017), also reported that the combined %RSD for all eight cannabinoids ranged from 2.5% to 5.2% and 0.28% to 5.5% for intra-day and inter-day measurements, respectively [31].

Accuracy

An evaluation of accuracy or bias was conducted for each cannabinoid at various concentration levels (2, 20, and 50 µg/mL) in both intra-day and inter-day measurements. The results revealed that the accuracy, represented by bias, was below 7.6% for intra-day and 10.4% for inter-day analyses (Table 3). In support of this findings, Zivovinic, S., *et al.* (2018), reported bias values lower than 10.3% for eight cannabinoids (CBDV, CBDA, CBGA, CBG, CBD, CBN, THC and THCA) in their study [32]. Similarly, Giese, M. W., *et al.* (2019) demonstrated that the bias for eight cannabinoids (THCA, CBGA, CBDA, THCVA, THC, CBD, CBG and THCV) was less than 11.8%, and the relative bias was also favourable. These studies further confirm the reliability and accuracy of the assay, suggesting that it can generate results that closely align with the true values [22].

Matrix effect

Matrix effect evaluations were conducted at various concentration levels (2, 20, and 50 µg/mL) for each cannabinoid. The results indicated that the matrix effect for all analytes fell within the range of $\leq 21.99\%$ (Table 3). In this study, the observed matrix effect percentages ranging from -10.38 to 21.99%. It is important to note that matrix effects within the range of $\leq 15\%$ are generally considered acceptable in analytical chemistry, if they are consistent and reproducible [33].

Table 2. The recovery of cannabinoids using the validated HPLC (Mean = 85-112%, SD $\leq$ 2%).

Cannabinoids	Sample number	Calculated concentration	Expected concentration	Recovery %
CBDV	1	1.479	1.665	88.828
	2	1.405		84.384
	3	1.89		113.513
	4	1.517		91.111
	5	1.599		96.036
	6	1.784		107.147
CBDA	1	12.073	15.243	79.203
	2	13.417		88.020
	3	14.338		94.062
	4	12.695		83.284
	5	11.871		77.878
	6	15.475		101.522
CBGA	1	7.629	8.553	89.196
	2	8.117		94.902
	3	8.808		102.981
	4	6.843		80.007
	5	7.309		85.455
	6	9.29		108.616
CBG	1	2.935	3.526	83.238
	2	3.258		92.399
	3	3.403		96.511
	4	2.997		84.997
	5	2.871		81.423
	6	4.281		121.412
CBD	1	7.135	8.358	85.367
	2	8.553		102.333
	3	8.96		107.202
	4	7.26		86.862
	5	6.973		83.429
	6	9.44		112.945
THCV	1	1.396	1.523	91.661
	2	1.446		94.944
	3	1.385		90.938
	4	1.72		112.934
	5	1.584		104.005
	6	1.266		83.125
D9-THC	1	68.362	89.211	76.629
	2	72.873		81.686
	3	79.104		88.670
	4	65.823		73.783
	5	67.562		75.732
	6	78.879		88.418
D8-THC	1	10.725	13.853	77.420
	2	11.672		84.256
	3	15.376		110.994
	4	12.006		86.667
	5	13.228		95.488
	6	14.39		103.876
CBC	1	2.468	2.965	83.237
	2	2.84		95.784
	3	2.845		95.952
	4	2.339		78.887
	5	2.899		97.774
	6	2.799		94.401
THCA	1	92.965	118.355	78.547
	2	107.684		90.983
	3	110.147		93.064
	4	89.816		75.886
	5	96.224		81.301
	6	108.44		91.622

Table 3. Precision, accuracy, and matrix effect results using HPLC (SD≤1.5%).

Repeats	%Precision (N=6)			%Precision (N=6)			%Accuracy (N=6)			%Accuracy (N=6)			%Matrix effect (N=6)		
	Intra-day			Inter-day			Intra-day			Inter-day					
Cannabinoids	Low (2 µg/mL)	Medium (20 µg/mL)	High (50 µg/mL)	Low (2 µg/mL)	Medium (20 µg/mL)	High (50 µg/mL)	Low (2 µg/mL)	Medium (20 µg/mL)	High (50 µg/mL)	Low (2 µg/mL)	Medium (20 µg/mL)	High (50 µg/mL)	Low (2 µg/mL)	Medium (20 µg/mL)	High (50 µg/mL)
CBDV	1.29	0.63		1.00	0.37	0.34	7.63	-2.07	-0.54	6.86	9.80	9.85	-2.08	-1.29	2.11
CBDA	2.89	0.81	0.74	2.82	0.72	0.45	0.89	2.54	2.06	-74.67	-2.92	4.44	13.37	3.45	2.77
CBGA	0.42	0.79	0.65	1.04	0.47	0.45	1.60	3.27	1.43	1.99	8.40	6.27	-5.50	-6.39	7.28
CBG	4.00	0.86	1.04	2.82	0.97	0.87	-0.14	4.92	-1.72	-6.16	6.88	8.22	-4.51	-8.04	2.63
CBD	6.63	0.78	0.88	4.91	0.53	0.43	1.93	-0.51	3.66	-30.80	7.66	9.62	-2.98	-2.51	2.62
THCV	5.92	0.57	0.80	5.21	0.58	0.59	-7.89	5.32	2.29	9.28	9.81	8.69	-2.58	-2.36	1.60
CBN	4.41	1.20	0.64	7.32	0.91	0.33	0.86	0.42	4.41	8.21	9.57	8.79	-6.85	-2.37	2.11
THC-d9	5.53	1.50	1.31	6.81	1.02	1.02	-4.03	2.49	2.25	-182.83	5.38	4.66	-6.99	7.17	9.05
THC-d8	7.28	0.64	0.44	8.73	1.20	0.38	6.60	-1.37	6.67	5.54	8.86	10.49	9.55	-10.38	-2.95
CBL	9.39	3.01	1.05	8.19	1.62	0.61	0.57	3.98	6.23	8.83	9.82	6.13	-3.83	-0.96	1.16
CBC	4.91	1.34	1.11	4.22	0.89	1.04	4.50	-7.63	1.50	-1.26	9.47	9.77	-10.17	-1.82	2.01
THCA	3.10	3.75	3.64	1.88	4.04	4.02	-13.78	1.01	3.41	-6.16	6.42	8.34	21.99	-2.79	5.10

Method validation for terpenes (GC-MS)

To assess the specificity of the analytical method, a solvent blank of methanol was injected, and no interfering peaks were observed at the targeted retention time region. Furthermore, six different concentrations of terpene standards (40, 20, 10, 4, 2 and 1 µg/mL) were injected, and distinct, well-separated

peaks were observed for each compound (Figure 4). This observation confirms the ability of the method to accurately differentiate and quantify individual terpenes without any interference from other components present in the sample matrix.

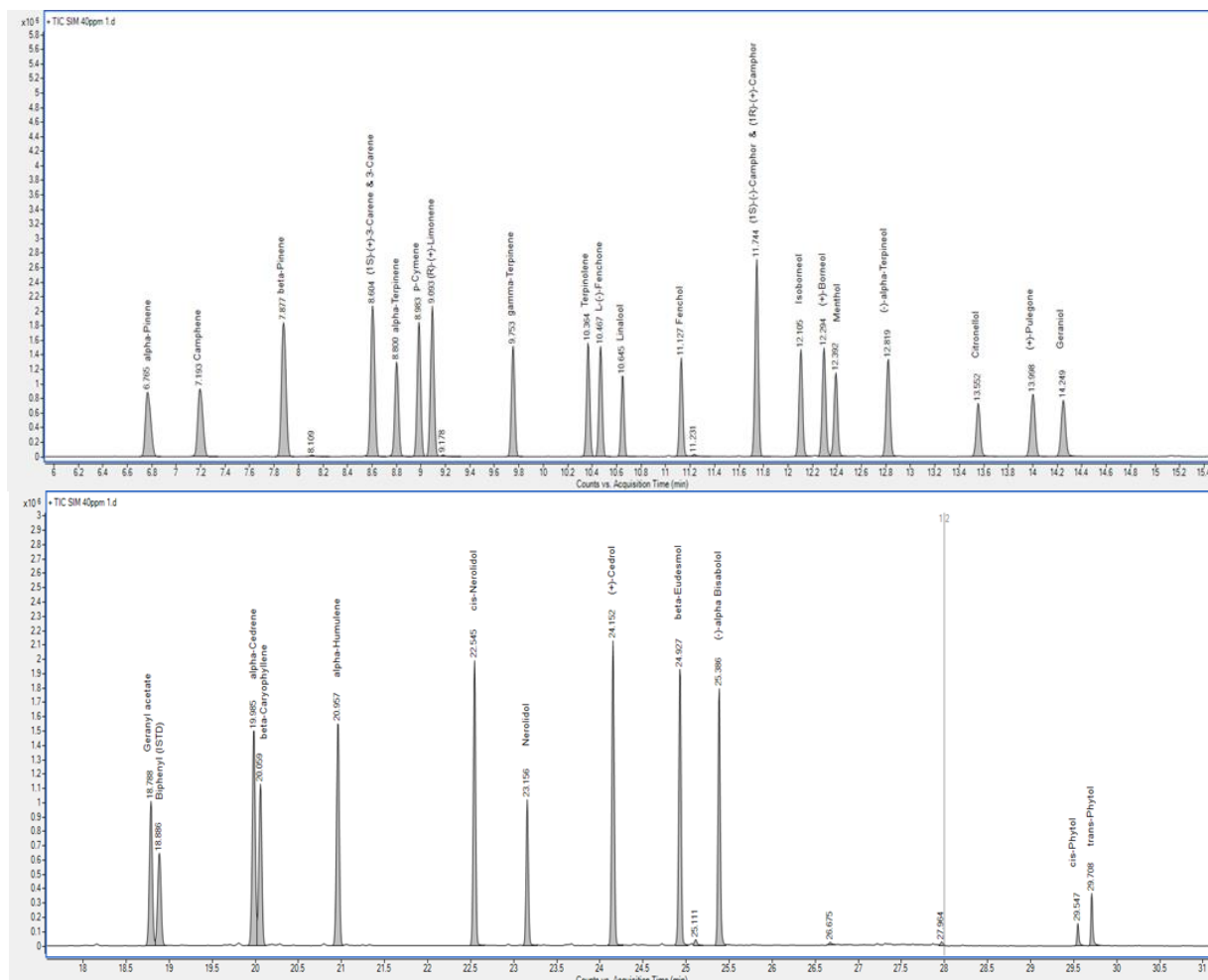


Figure 4. GC/MS chromatogram of 32 terpenes with concentration of 40 µg/mL.

Linearity

The correlation coefficients obtained for the analysis of 30 terpenes in this study ranged from 0.995 to 0.999 within the concentration range of 1-40 µg/mL. These high correlation coefficients indicate a strong linear relationship between the concentration of the terpenes and their corresponding analytical signals. In a previous study by Ibrahim, E. A., *et al.* (2019), a GC-MS method was validated for the analysis of 10 terpenes. The calibration curves for β -myrcene, α -pinene, β -pinene, terpinolene, and linalool exhibited linearity in the concentration range of 0.75-75 µg/mL. For limonene, α -terpineol, β -caryophyllene, and α -humulene, the linear range was determined to be 0.75-70 µg/mL.

Caryophyllene oxide, on the other hand, displayed linearity in the range of 1.0-50 µg/mL. Importantly, all terpenes showed r^2 values exceeding 0.99, indicating an excellent fit of the calibration curves [2]. Similarly, a recent study conducted by Myers, C., *et al.* (2021) evaluated the calibration performance of 23 terpenes of interest. The average r^2 value obtained for these terpenes was 0.993, demonstrating a strong linear correlation between the concentration and the analytical signals. For 21 out of the 23 terpenes, the r^2 value was ≥ 0.990 , indicating a high level of linearity. The remaining two compounds, geraniol and trans-nerolidol, had an r^2 value of 0.988 [29]. The finding supports Ibrahim, E. A., *et al.* (2019) and Myers, C., *et*

al. (2021) support the results observed in this study, confirming the successful validation of the analytical methods and the achievement of strong linear correlations between terpene concentrations and their corresponding analytical signals.

LOD and LOQ

LOD and LOQ are important parameters in analytical methods as they indicate the lowest concentrations at which analytes can be reliably detected and quantified. The LOD and LOQ results for terpenes are presented in

Table 4. The LOD values ranged from 0 to 0.05 µg/mL, while the LOQ values ranged from 0.01 to 0.22 µg/mL. Comparing these results with previous studies, Jin *et al.* (2017) validated a GCMS method for 14 terpenes and reported LODs ranging from 0.82 to 3.69 µg/mL, and LOQs ranging from 2.47 to 11.2 µg/mL for all terpenes [27]. Myers *et al.* (2021) and Fernández *et al.* (2023) also reported LOQ values between 0.017 to 0.129 µg/mL and less than 2.4 µg/mL, respectively [34, 35].

Table 4. Linearity, LOD and LOQ of terpenes using GC-MS (SD ≤ 3%).

Terpenes	Coefficient of determination (R ²) (N=6)	LOD (µg/mL) (N=6)	LOQ (µg/mL) (N=6)
alpha-Pinene	0.997	0.067	0.222
Camphene	0.996	0.066	0.199
beta-Pinene	0.995	0.063	0.209
(1S)-(+)-3-Carene & 3- Carene	0.996	0.061	0.203
alpha-Terpinene	0.998	0.001	0.004
p-Cymene	0.996	0.059	0.196
(R)-(+)-Limonene 7 Limonene	0.996	0.056	0.186
gamma-Terpinene	0.998	0.001	0.005
Terpinolene	0.998	0.001	0.003
L-(-)-Fenchone	0.997	0.002	0.008
Linalool	0.998	0.001	0.003
Fenchol	0.999	0.001	0.005
(1S)-(-)-Camphor & (1R)-(+)-Camphor	0.996	0.002	0.007
Isoborneol	0.998	0.003	0.009
(+)-Borneol	0.998	0.003	0.012
Menthol	0.999	0.001	0.003
(-)-alpha-Terpineol	0.992	0.001	0.002
Citronellol	0.999	0.001	0.002
(+)-Pulegone	0.999	0.001	0.002
Geraniol	0.999	0.000	0.001
Geranyl acetate	0.999	0.000	0.001
alpha-Cedrene	0.998	0.002	0.006
beta-Caryophyllene	0.997	0.000	0.001
alpha-Humulene	0.999	0.002	0.007
cis-Nerolidol	0.999	0.002	0.005
Nerolidol	0.999	0.001	0.002
(+)-Cedrol	0.999	0.001	0.004
beta-Eudesmol	0.998	0.001	0.004
(-)-alpha Bisabolol	0.999	0.001	0.003
Phytol	0.998	0.001	0.005

Recovery

The recovery of terpenes indicates the accuracy and efficiency of the extraction and analysis process. In this study, the recovery percentages of terpenes ranged from 79% to 91%, with terpinolene, phytol, and geraniol showing lower recoveries of 66%, 71%, and 72% respectively (Table 5). Comparing these results with previous studies, Ibrahim *et al.* (2019) reported the average recoveries of specific terpenes from indoor cultivated samples, with values ranging from 95% to 103.3%, except for terpinolene which exhibited a recovery similar to our findings [19]. Jin *et al.* (2017)

evaluated the recovery of 14 terpenes and reported values ranging from 93% to 104.8% [27]. Despite the lack of specific literature on cannabis terpenes, the lower recovery observed in geraniol and phytol can still be considered acceptable, as it exceeds the threshold of 70% outlined in the guidelines provided by the Food and Agriculture Organisation of the United Nations [36].

Table 5. The recovery of 30 terpenes using the validated GC-MS ($SD \leq 5\%$).

Terpenes	Calculated concentration	Expected concentration	% Recovery (N=3)
alpha-Pinene	15.34	19.80	86.07
Camphene	15.58	19.92	86.90
beta-Pinene	30.88	41.84	82.01
(1S)-(+)-3-Carene & 3-Carene	30.07	40.39	82.72
p-Cymene	14.94	20.41	81.36
(R)-(+)-Limonene 7 Limonene	15.10	20.10	83.47
gamma-Terpinene	15.95	19.92	88.95
Terpinolene	12.03	20.06	66.63
L-(-)-Fenchone	16.22	19.67	91.61
Linalool	14.61	19.66	82.56
Fenchol	15.49	18.89	91.09
(1S)-(-)-Camphor & (1R)-(+)-Camphor	30.73	39.96	85.46
Isoborneol	16.11	19.92	89.87
(+)-Borneol	14.43	20.15	79.60
Menthol	15.01	18.78	88.82
(-)-alpha-Terpineol	14.44	20.08	79.90
Citronellol	15.86	20.33	86.71
(+)-Pulegone	16.16	19.98	89.88
Geraniol	13.80	21.03	72.90
Geranyl acetate	15.49	19.98	86.10
alpha-Cedrene	15.30	18.98	89.54
beta-Caryophyllene	15.42	20.48	83.65
alpha-Humulene	15.25	18.80	90.13
cis-Nerolidol	14.65	20.46	79.52
Nerolidol	15.32	19.95	85.28
(+)-Cedrol	16.04	20.00	89.11
beta-Eudesmol	14.82	20.57	80.06
(-)-alpha Bisabolol	15.71	19.81	88.15
Phytol	14.64	22.59	71.99

Precision

The assessment of precision in terpene analysis involved evaluating their performance at various concentration levels (1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, and 20 $\mu\text{g/mL}$) for both intra-day and inter-day variations. The obtained results demonstrated that the precision of each terpene was consistently below 8.1% for intra-day measurements and 10.7% for inter-day measurements. These values suggest a high level of precision and indicate that the analytical method employed can generate reliable and consistent results. Comparing these findings to the study conducted by Jin *et al.* (2017), where the method precisions (%RSD) for 14 terpenes were less than 2.1%, reveals that both studies achieved a commendable level of precision. They also reported inter-day injection precisions below 1.34%, further highlighting the robustness and consistency of their analytical method [27]. The differences between Jin *et al.* (2017) and this study are in both GC system (injection volume, injection ratio and run time) and sample preparation techniques such as using different solvents and different extraction method (Maceration). Little (2016) established acceptance criteria for analytical methods, stating that the acceptable intermediate precision (IP) should be equal to or less than 25% [37].

Accuracy

In the present study, each terpene was evaluated for accuracy at various concentration levels (1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, and 20 $\mu\text{g/mL}$). The results demonstrated that the accuracy, or bias, was consistently below 10.2% for all the terpenes analysed. These findings indicate that the measured values closely align with the true values, suggesting a high level of accuracy in the analytical method employed. Comparing these results to the study conducted by Fernández *et al.* (2023), where the accuracy for seven terpenes was reported to be less than 15%, reveals that both studies achieved satisfactory accuracy levels [35]. Additionally, Giese *et al.* (2019) validated 17 terpenes' accuracy using GC-MS, showing accuracy below 5.2% [22]. It is worth noting that the acceptable criteria for accuracy can vary depending on the specific analysis and the compounds being studied. According to Little (2016), an accuracy level of 10% or lower is considered excellent.

Matrix effect

Matrix effect evaluations were carried out at different concentration levels (1 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, and 20 $\mu\text{g/mL}$) for each terpene. The matrix effect for all terpenes varied between -72.96% and 20.24%, as shown in Table 6. Unfortunately, the literature has limited papers discussing the matrix effect in the

Table 6. Precision, accuracy, and matrix effect of terpenes using GC-MS.

Terpenes	%Precision (N=6)			%Precision (N=6)			%Accuracy (N=6)			%Matrix effect (N=6)		
	Intra-day			Inter-day								
	Low (1 µg/mL)	Medium (10 µg/mL)	High (20 µg/mL)	Low (1 µg/mL)	Medium (10 µg/mL)	High (20 µg/mL)	Low (1 µg/mL)	Medium (10 µg/mL)	High (20 µg/mL)	Low (1 µg/mL)	Medium (10 µg/mL)	High (20 µg/mL)
alpha-Pinene	3.69	2.11	4.44	5.52	9.82	1.10	-28.17	9.25	6.48	7.76	-12.15	-2.05
Camphene	3.51	1.86	4.09	5.52	9.79	1.22	-7.76	10.25	9.31	20.24	-3.24	-8.17
beta-Pinene	3.35	1.74	3.86	5.02	6.35	1.48	-86.08	7.25	4.45	8.49	-19.35	-9.71
(1S)-(+)-3-Carene & 3-Carene	3.22	1.77	3.59	5.04	6.49	1.65	-24.11	8.26	4.33	7.23	-7.72	-5.19
p-Cymene	8.16	5.97	7.25	1.34	9.48	3.92	-6.42	7.96	6.59	5.88	-5.25	11.15
(R)-(+)-Limonene 7 Limonene	2.98	1.24	2.86	4.28	3.44	1.89	-88.25	9.24	-4.85	-5.62	-14.25	-7.29
gamma-Terpinene	3.40	1.26	3.40	5.17	6.64	1.69	-23.32	7.12	3.13	-0.63	-8.21	-4.06
Terpinolene	2.93	1.61	3.52	5.87	9.16	1.89	7.25	7.39	6.47	12.50	-2.94	-3.44
L-(-)-Fenchone	5.07	3.03	4.40	2.28	5.72	2.52	-99.59	7.34	-3.65	9.57	5.42	14.53
Linalool	2.48	1.70	2.45	5.56	10.74	2.40	-27.75	-2.53	-2.77	5.14	-3.19	-6.20
Fenchol	4.77	2.36	3.11	6.72	3.38	2.96	-93.89	-4.73	-13.39	10.92	-5.23	10.17
(1S)-(-)-Camphor & (1R)-(+)-Camphor	2.90	3.03	3.06	5.33	8.48	2.73	-10.20	-3.99	-3.09	-5.89	-2.54	0.03
Isoborneol	2.35	2.01	2.30	5.27	7.21	2.43	-33.04	0.58	-7.55	-5.92	-7.01	-2.38
(+)-Borneol	3.63	2.69	3.27	5.89	9.89	2.92	-12.82	-3.16	-3.21	0.16	-1.46	3.63
Menthol	4.49	2.95	2.86	5.58	3.99	3.28	-99.05	-0.86	-11.02	-11.23	-8.44	4.90
(-)-alpha-Terpineol	3.25	2.95	3.18	6.40	9.20	2.94	6.97	-2.87	-3.44	21.75	0.89	3.66
Citronellol	2.40	2.60	2.49	5.28	4.03	3.05	-99.45	-2.91	-10.75	-19.73	-9.93	3.59
(+)-Pulegone	1.42	1.92	2.69	7.51	10.15	3.59	2.36	-6.18	-0.45	2.92	1.14	13.47
Geraniol	2.07	2.19	2.56	6.55	9.96	3.02	-11.39	-4.54	-2.16	-1.71	-1.42	3.31
Geranyl acetate	6.33	1.09	1.79	3.51	5.33	3.77	-97.28	-20.25	-11.42	-12.26	-6.69	-1.18
alpha-Cedrene	0.78	2.21	2.24	5.43	13.49	3.47	-45.29	-11.37	-1.56	-3.97	-2.08	1.66
beta-Caryophyllene	2.96	2.65	2.02	5.58	8.14	2.67	-11.44	-2.58	-2.65	-8.19	-4.96	-3.13
alpha-Humulene	3.25	2.56	2.42	5.46	3.43	2.85	-95.54	0.09	-10.62	-9.92	-51.49	-3.34
cis-Nerolidol	3.19	2.78	2.64	6.21	9.61	2.83	7.82	-3.11	-2.94	0.48	-18.66	-9.70
Nerolidol	2.45	3.50	3.29	5.90	8.48	3.64	-80.75	-3.34	-4.27	-21.94	-3.49	12.92
(+)-Cedrol	1.59	3.21	3.10	8.57	10.79	3.96	-49.94	-16.22	-7.33	-72.96	-4.38	10.02
beta-Eudesmol	2.23	2.94	2.54	5.30	8.02	3.19	2.47	-2.90	-3.46	-6.36	-6.74	1.56
(-)-alpha Bisabolol	3.01	3.24	2.95	5.72	4.34	3.78	-98.95	-1.07	-9.75	1.75	-11.75	4.62
Phytol	2.80	3.08	2.15	5.72	10.09	3.75	-21.76	-5.19	-0.87	9.68	-17.59	-3.57

context of the cannabis plant, primarily due to the difficulty in obtaining a blank matrix of cannabis plant without terpenes. Myers *et al.* (2021) highlighted that previous studies did not account for matrix effects as they relied on solvent-based calibrations. Given the complexity and impurities in the cannabis matrix, this approach could lead to inaccurate reporting. To address this, they utilised hops as a blank matrix to validate the GC-MS method for terpenes [34]. Steiner *et al.* (2020) indicated that an acceptable matrix effect should be below 20% [38]. This shows the results of current study is within the normal range and it can be related to extraction method for obtaining blank matrix.

Conclusion

In conclusion, this study validates HPLC and GC-MS methods for quantifying cannabinoids and terpenes, selected after extensive literature review. Sample preparation utilised an optimised UAE method, ensuring efficient extraction with minimal analyte loss. Meticulous assessment of parameters confirmed the methods' robustness and accuracy. The HPLC method exhibited exceptional linearity, with the correlation coefficient approaching 1, indicating a strong linear relationship between the analytes' concentrations and their corresponding peak areas. Additionally, the LOD and LOQ were found to be lower than the lowest standard, indicating the sensitivity of the method. Furthermore, the recovery of cannabinoids in the HPLC method fell within the recommended range of 70-120%, demonstrating the efficiency of the extraction and analytical processes. The precision and accuracy of the HPLC method were also evaluated, with both inter-day and intra-day variations yielding values below 10%, indicating good reproducibility and accuracy. Regarding GC-MS, the linearity of the method was deemed acceptable, as the calibration curves demonstrated a satisfactory correlation between terpene concentrations and their corresponding peak areas. The LOD and LOQ values for all terpenes were below the lowest standard, highlighting the method's sensitivity in detecting even trace amounts of these compounds. The recovery of terpenes ranged between 80-90%, indicating the efficiency of the extraction procedure. The precision and accuracy were also within the desirable range, with variations below 10%, further attesting to the method's reliability and accuracy. Additionally, the evaluation of the matrix effect revealed values mainly below 9.55% for HPLC and below 15% for GC-MS, indicating minimal interference from matrix components on the analyte's signal.

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Conflict of interest

The authors declare no conflicts of interest.

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