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Determination of Terpenoid Profile in Dry Cannabis Flowers and Extracts Obtained from Different Cannabis Varietes

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of this study was to determine the terpenoid profile in dried cannabis flowers obtained from different varieties of cannabis plant and in cannabis extracts in order to investigate quantity of terpenes lost during extraction and purification process.
Methods: GC/MS method for determination of terpenes was verified. The concentration of terpenes was determined in dry flowers as raw material and in decarboxylated and distillated cannabis extracts, using the same GC/MS analytical method. The extraction was performed using 96% ethanol as a solvent.

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Results: The obtained results indicate that dry cannabis flowers from different cannabis plant can be distinguished only by their terpenoid profile. The use of standardized cannabis-based extracts can be confirmed by determination of terpenoid profile. The purification process of the cannabis extracts removes terpenes. The percentage of major terpen beta-Myrcene decreased from 68% in dry flower to 15% in decarboxylated and, 1.9% in distillated cannabis oil after purification. The percentage of second major terpene alpha-Pinene decreased from 15% in dry flower to 5% in decarboxylated and, 0.7% in distillated cannabis oil after purification.
Conclusion: Terpenes act synergistically with cannabinoids. Following the monograph for quality testing of cannabis extracts in the German Pharmacopoeia, the purification process is necessary to achieve a final concentration of cannabinoids (Tetrahydrocannabinol) of more than 95% in the final active pharmaceutical ingredient. The purification process removes terpenes that have proven synergistically pharmacological effects with cannabinoids.

Keywords: Terpenes; terpenoid profile; cannabis extracts; GC/MS determination; fingerprint.

1. INTRODUCTION

Cannabis sativa L. (Cannabaceae) is the frequently used plant, yet notorious and controversial, but considered to have therapeutic potential [1]. Several cannabis-based medicines are now available for the treatment of various pathological conditions such as treatment of pain in cancer patients, treatment of nausea and vomiting induced by chemotherapy, loss of appetite and treatment of cachexia in patients with cancer and acquired immunodeficiency syndrome (AIDS), treatment of neuropathic and chronic pain and spasticity in multiple sclerosis [2-6].

Cannabis is a plant that contains more than 1,000 different chemical ingredients, which vary depends on the chemotype (chemical phenotype) of the strain. Chemotypes denote plants of the same genus that are practically identical in appearance but produce essential oil containing different major ingredients that vary within one botanical strain [7].

1.1 Importance of Terpenes / Terpenoids

An essential oil (extract) derived from cannabis plats primarily contains cannabinoids which are the main carriers of pharmacological effects and terpenes / terpenoids, which act synergistically with cannabinoids in exhibiting a pharmacological effect. Terpenes / terpenoids are responsible for the characteristic aroma of cannabis extracts.

Terpenes / terpenoids itself have a wide range of pharmacological actions, such as antifungal, antiviral, anticancer, anti-inflammatory, antihyperglycemic, antiparasitic, antioxidant and antimicrobial. For example, monoterpene myrcene which is the smallest terpene, has antipsychotic, antioxidant, analgesic, anti-inflammatory, sedative, muscle relaxant and anticancer effects [8–10]. Caryophyllene has gastroprotective, analgesic, anticancer, antifungal, antibacterial, antidepressant, anti-inflammatory, antiproliferative, antioxidant, and neuroprotective effects [11]. α-pinene has antibacterial, anti-inflammatory, broncho dilatory, antiseptic and gastroprotective pharmacological effects, while β-pinene has only an antiseptic effect [12]. Linalol is a terpene that acts as a sedative, antipsychotic, anticonvulsant, anxiolytic, anesthetic, antidepressant, analgesic, antiepileptic and antineoplastic [8]. Terpineol has an antioxidant, antimicrobic, and relaxing effect, while caryophyllene has analgesic, anticancer and antifungal effects [13]. Other terpenes like phellandrene and ocimene, has only an antifungal effect and they are used to treat different digestive disorders [14-15]. Camphene helps in treatment of cardiovascular diseases, while guaiol has an antitumor effect [16]. α-humulene has antibacterial, anti-inflammatory, and antitumor effects [17], nerolidol antiparasitic [18, 19], and citral has an antifungal, antimicrobial, antiproliferative, cytotoxic, anticancer, and antitumor effect [20–25].

Due to their synergistic effect, several therapeutic approaches based on the combined use of cannabinoids and terpenes have recently been developed [26-29]. Considering that different terpenes have different pharmacological effects, standardization of these products, which can be very heterogeneous [30] depending on the variety of plants from which they are obtained, is an important prerequisite for confirming the quality and expected pharmacological effect [31]. This is especially important if we consider that the terpenoid profile

is a fingerprint or a specificity that is characteristic of each variety.

Therefore, our goal was to develop or verify the GC/MS method for determination of 35 terpenes and to monitor their content in a cannabis dry flower from different varieties as well as in cannabis extracts obtained after extraction process of the same flowers in order to investigate quantity of terpenes lost during extraction and purification process.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Standards for cannabis terpenes as a Reference material were supplied by Restek and Sigma Aldrich (Table 1). Helium gas was supplied by Messer.

2.2 Apparatus

Terpene analysis were performed on a GCMS-QP2010SE single quadrupole mass spectrometer with static headspace (HS-20) with loop and autosampler for sample introduction.

2.3 Instrument Operating Conditions and Method Parameters

Instrument operating conditions and method parameters [32] are shown in Table 2. Verification of the method was fully implemented.

2.4 Standard Solutions and Calibration Curves

Three sets of standards were used to obtain a more complete terpene profile. Standard one, purchased from Restek in a 2500 µg/mL stock solution, and Standard two and three, purchased from Sigma Aldrich (SPEX mix A and SPEX mix B) in a 100 µg/mL stock solution. 35 different terpenes were identified and quantified in total.

Full evaporation headspace technique (FET) was used for quantification. A five-point calibration curves were created from the Restek terpene standard with concentration ranging from 78-2500 µg/mL and Sigma Aldrich terpene standards (mix A and mix B) with concentrations ranging from 12.5-100 µg/mL. An aliquot of 10µL of the standard was placed in a 10mL headspace vial and capped. All points on the calibration curve were run in replicates of six.

2.5 Verification of the Method

The proposed method was verified according to the guidelines set by the International Conference of Harmonization for validation of analytical procedures [33, 34]. The precision and reproducibility of the proposed method were evaluated by performing six replicate analyses of the standard solutions for five different concentrations. Relative standard deviations were calculated to obtain the precision of the method. The full mass scan was done for all standard mix solutions to conform the specificity / selectivity of the method. To confirm the linearity of the method standard solutions in at least five different concentrations was prepared for all analytes. The limit of detection and limit of quantification for each analyte were calculated from standard error, slope, and analyte response.

2.6 Extraction Process

The extraction process was performed using 96% ethanol as a solvent. Maceration was performed in a cold chamber (refrigerator at -20^{o}C). The duration of the maceration was 30 minutes in total. Stirring was done on every 10 minutes. After maceration was completed, the macerated material (cannabis flowers) was manually squeezed with a stainless-steel strainer. The resulting macerate was filtered. After that the ethanol was evaporated. After evaporation of the ethanol, the obtained crude oil was decarboxylated by heating until the temperature of the crude extract reached 125-130^{o}C. After decarboxylation, additional purification was performed to obtain an extract (distillated cannabis oil) having more than 95% THC according to the monograph in the German Pharmacopoeia.

2.7 Sample Preparation (dry flower or cannabis extract)

FET was used for quantitation. 30mg of the dry cannabis flower or cannabis extract (decarboxylated or distillated oil) were weighed into a headspace vial and capped. Calculations of the quantity of different terpenes were done using calibration curve for each terpen separately. Analyze was done on nine different

Table 1. Standards for cannabis terpenes used as a Reference material

Substance name	Lot No:	Manufacturer	Expiration date
Cannabis terpenes standard #1	A0155278	Restek	11.2021
Cannabis Terpene Mix A	LRAC3834	Sigma Aldrich	09.2022
Cannabis Terpene Mix B	LRAC7120	Sigma Aldrich	09.2023

Table 2. Instrument operating conditions and method parameters

Head Space	**HS-20 Loop Model**
Operation Mode	Static headspace with loop
Sample	10 µl sample volume
	10 ml headspace vial
Equilibration	30 minutes at 150^{o}C
Sample Loop	1 ml loop
	Vial pressurization 1.00 min, equilibration 0.20 min
	Loop load time 1.00 min, equilibration 0.20 min
	Injection time 1.0 min
Sample Pathway Temperature	150^{o}C
Transfer Line Temperature	150^{o}C
Gas Chromatogram	**GC-2010 Plus**
Injection	Spliit injection from HS-20, with 50:1 split ration
Column	Rxi-624 Sil MS 30.0 m x 0.25 mm x 1.40 µm
	Helium carrier gas
	Constant linear velocity, 47.2 cm/sec
	Column Flow 1.64 ml/min
	Purge Flow 3.0 m/min
Oven program	80^{o}C, hold 1.0 min
	12^{o}C/min to 150^{o}C, hold 1.0 min
	9^{o}C/min to 250^{o}C, hold 1 min
	Total GC run time 19.94 min
	Total cycle time 24 min
Detector	**GCMS-QP2010 SE**
Operating mode	Selected Ion Monitoring (SIM) and SCAN
Ion Source	200^{o}C, EI mode, 70eV
Solvent Cut Time	2 min
MS Interface	300^{o}C

strains of cannabis plant and two decarboxylated and two distillated cannabis extracts obtained from two different strains of cannabis plant. The extraction was performed using 96% ethanol as a solvent through maceration.

3. RESULTS AND DISCUSSION

3.1 Verification of the Method

The precision and reproducibility of the proposed method were evaluated by performing six replicate analyses of the standard solutions for five different concentrations used for creation of calibration curves. Relative standard deviations (RSD) were calculated for each terpene. RSD for each terpene in each concentration after 6 replicate determinations was lower than 7% [35].

The typical chromatograms and calibration curves of the standard solutions of each terpen are shown in Figure 1-1, Figure 1-2 and Figure 1-3. Coefficient of correlation was greater than 0.99.

The full mass scan was done for all standard mix solutions to conform the specificity / selectivity of the method. At least 2 qualifier ions were used for identification and one quantifier ion for quantification. The results are shown in Table 3.

Limit of detection / Limit of quantification were calculated from standard error, slope, and analyte response. The results calculated as numerical (absolute) value from the calibration curve are shown in Table 4.

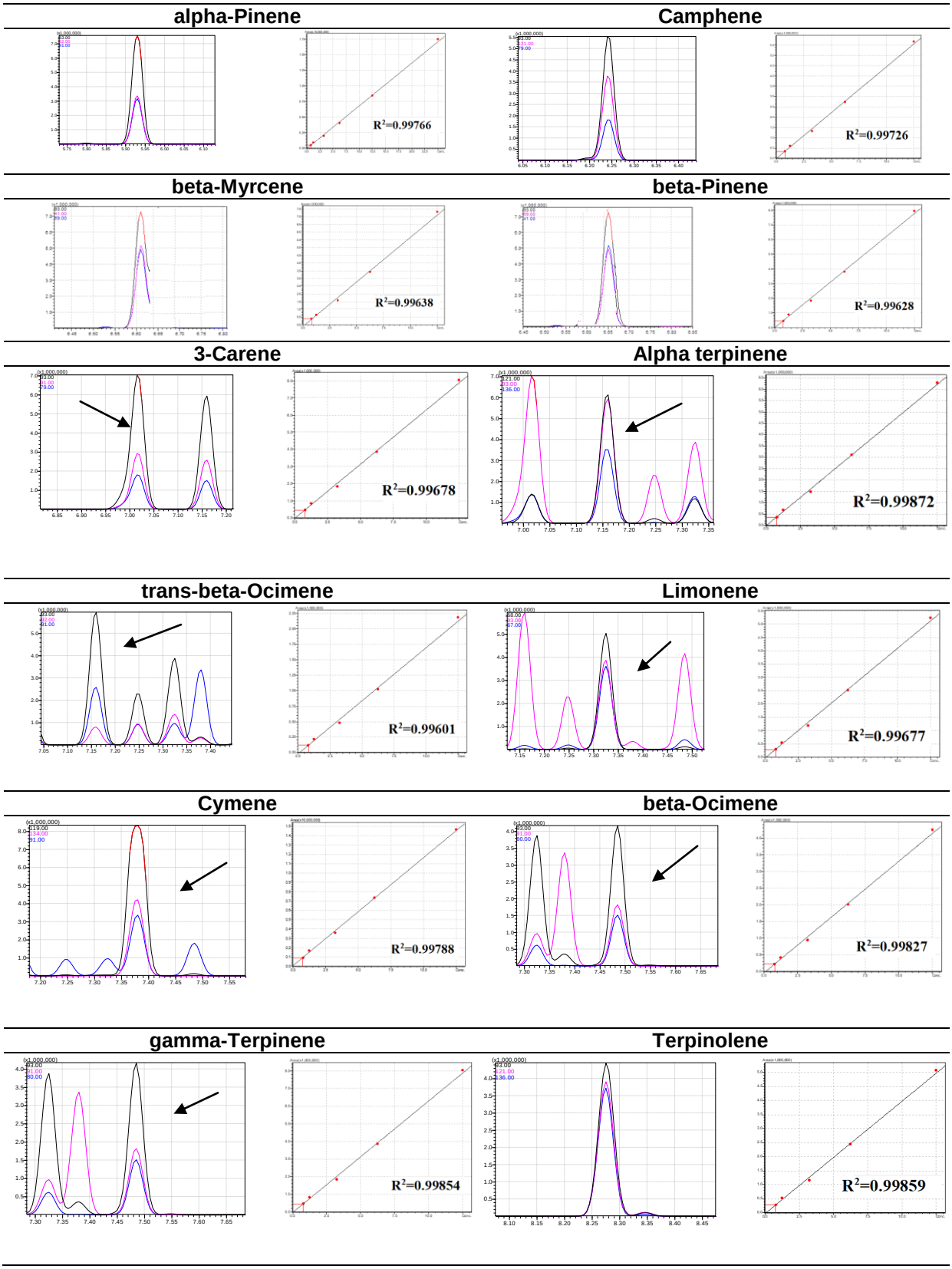

Fig. 1-a. Typical chromatograms (GS/MS) and calibration curves of the standard solutions for terpene testing

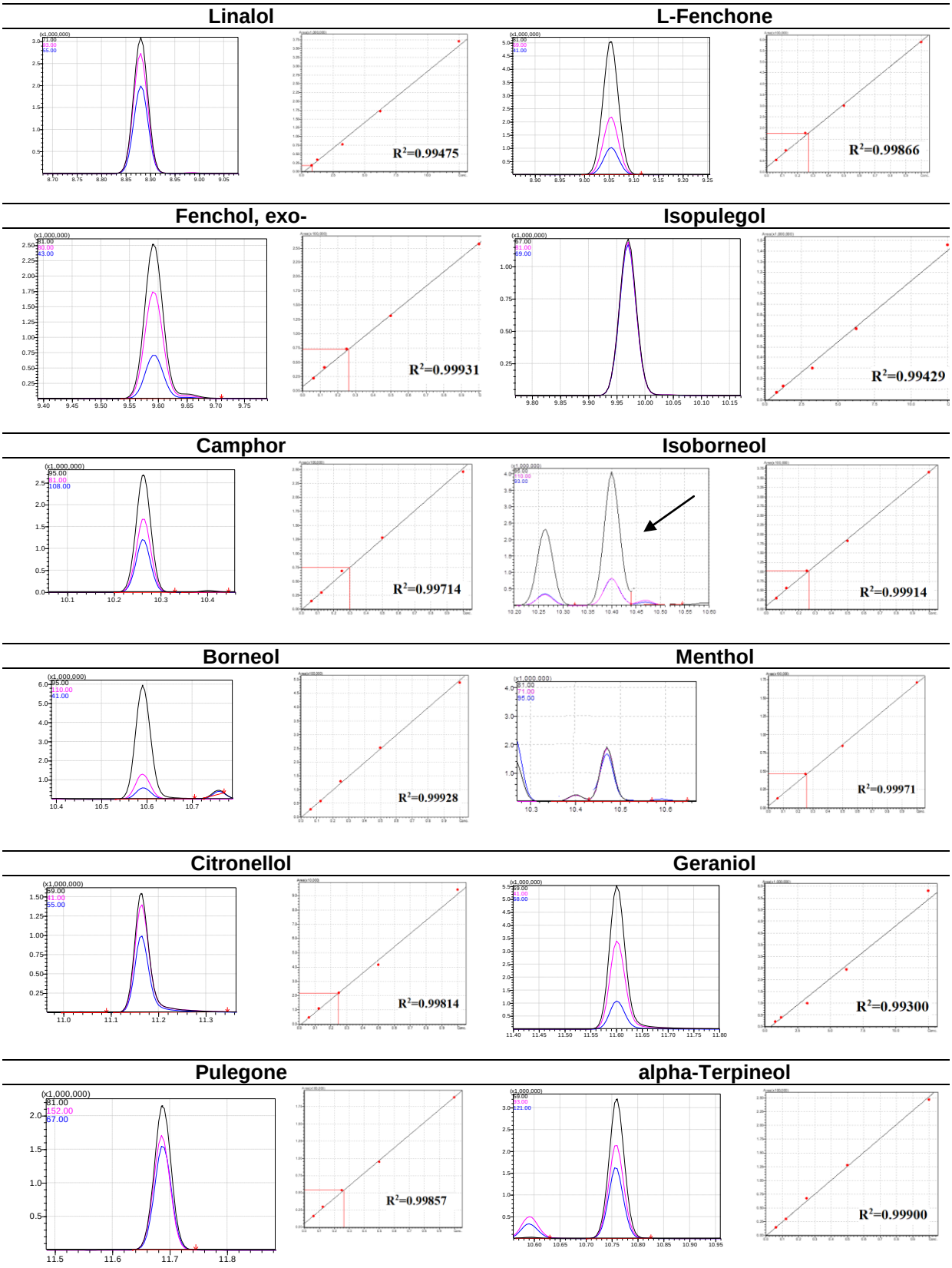

Fig. 1-b. Typical chromatograms (GS/MS) and calibration curves of the standard solutions for terpene testing

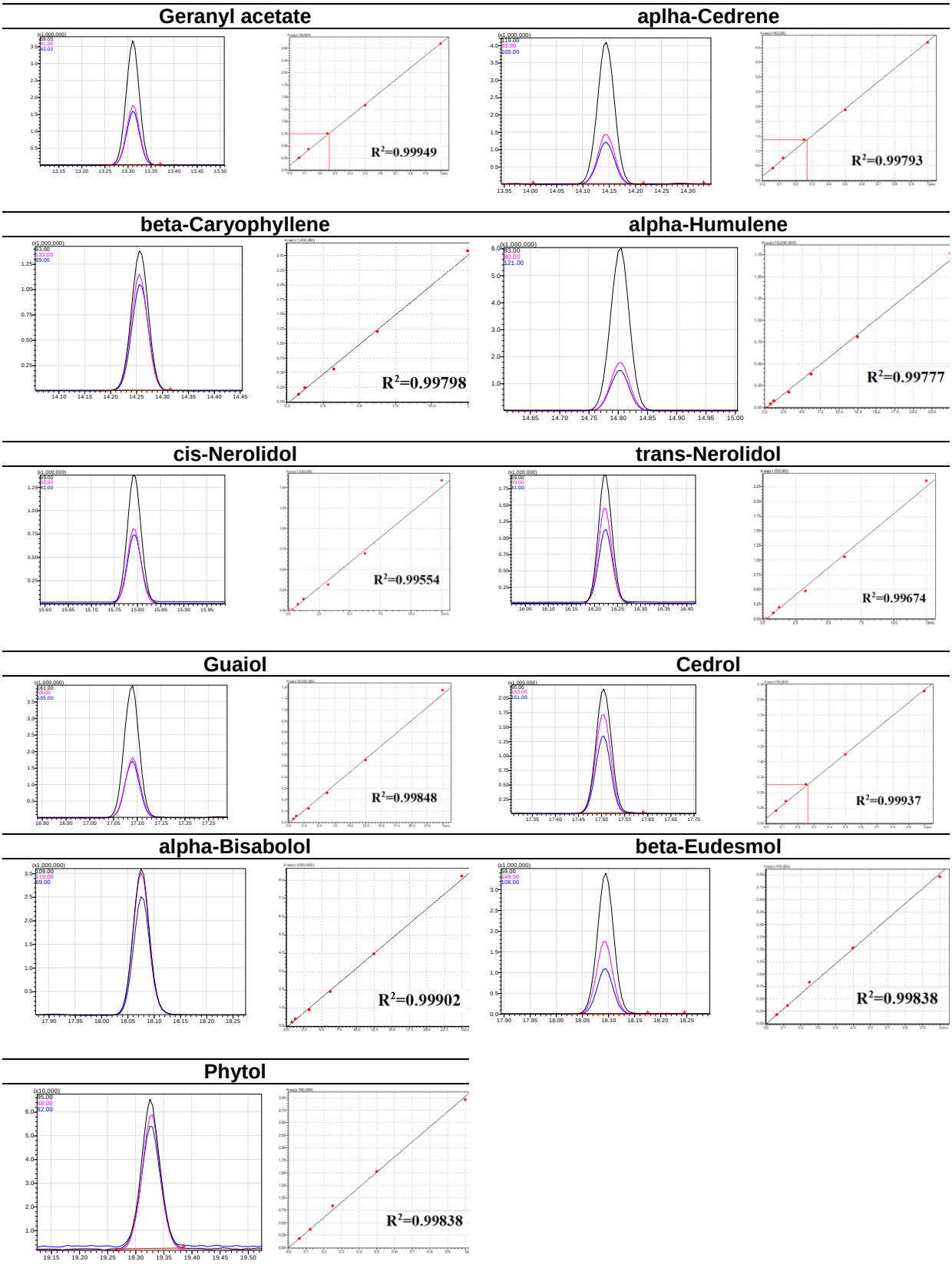

Fig. 1-c. Typical chromatograms (GS/MS) and calibration curves of the standard solutions for terpene testing

Table 3. Retention time and analyte transition for different terpenes

Terpene	Retention time (min)	Precursor Ion	Product Ion (Quantifier)	Product Ion (Qualifier)
alpha-Pinene	5.93	93	92	91
Camphene	6.24	93	121	79
beta-Myrcene	6.61	93	41	69
beta-Pinene	6.65	93	69	41
3-Carene	7.015	93	91	79
Alpha terpinene	7.16	121	93	136
trans-beta-Ocimene	7.245	93	92	91
Limonene	7.325	68	93	67
Cymene	7.38	119	134	91
beta-Ocimene	7.485	93	91	80
gamma-Terpinene	7.76	93	91	80
Terpinolene	8.275	93	121	136
Linalol	8.88	71	93	55
L-Fenchone	9.055	81	69	41
Fenchol, exo-	9.59	81	80	43
Isopulegol	9.97	67	81	69
Camphor	10.265	95	81	108
Isoborneol	10.4	95	110	93
Menthol	10.47	81	71	95
Borneol	10.59	95	110	41
alpha-Terpineol	10.76	59	93	121
Citronellol	11.165	96	41	55
Geraniol	11.6	69	41	68
Pulegone	11.685	81	152	67
Geranyl acetate	13.31	69	41	43
Alpha-Cedrene	14.145	119	93	105
beta-Caryophyllene	14.255	93	133	69
alpha-Humulene	14.805	93	80	121
cis-Nerolidol	15.79	69	93	41
trans-Nerolidol	16.22	69	93	41
Guaiol	17.09	161	59	105
Cedrol	17.505	95	150	151
alpha-Bisabolol	18.075	109	119	69
beta-Eudesmol	18.095	59	149	108
Phytol	19.325	95	68	82

Table 4. Limit of detection / Limit of quantification of different terpenes, calculated as numerical (absolute) value from the calibration curve

Terpene	Limit of Detection (µg/mL)	Limit of Quantification (µg/mL)
alpha-Pinene	0.544	1.649
Camphene	0.445	1.349
beta-Myrcene	0.547	1.658
beta-Pinene	0508	1.539
3-Carene	0.478	1.449
Alpha terpinene	0.387	1.174
trans-beta-Ocimene	0.573	1.738
Limonene	0.483	1.464
Cymene	0.327	0.993
beta-Ocimene	0.521	1.580
gamma-Terpinene	0.454	1.376
Terpinolene	0.446	1.35
Linalol	0.661	2.003

Terpene	Limit of Detection (µg/mL)	Limit of Quantification (µg/mL)
L-Fenchone	0.031	0.094
Fenchol, exo-	0.019	0.060
Isopulegol	0.691	2.095
Camphor	0.038	0.117
Isoborneol	0.026	0.079
Menthol	0.030	0.091
Borneol	0.028	0.086
alpha-Terpineol	0.032	0.097
Citronellol	0.060	0.182
Geraniol	1.140	3.456
Pulegone	0.024	0.074
Geranyl acetate	0.019	0.058
Alpha-Cedrene	0.027	0.081
beta-Caryophyllene	0.584	1.770
alpha-Humulene	0.929	2.816
cis-Nerolidol	0.942	2.857
trans-Nerolidol	0.777	2.355
Guaiol	0.687	2.082
Cedrol	0.019	0.060
alpha-Bisabolol	0.482	1.461
beta-Eudesmol	0.038	0.116
Phytol	0.098	0.297

Table 5. Terpenoid profile of different cannabis strains, calculated as percentage of total terpenes

Cannabis strain	BB*	AK*	WW*	HE*	SG*	LS*	GE*	FC*	AFG*
Terpene									
alpha-Pinene	10.997	16.314	15.287	11.487	1.453	2.682	4.917	2.869	5.611
Camphene	0.396	0.360	0.303	0.508	0.426	0.455	0.653	0.995	0.802
beta-Myrcene	62.998	55.347	68.887	48.943	38.367	11.562	10.048	11.540	17.471
beta-Pinene	3.678	7.414	6.438	3.057	0.197	4.096	4.503	4.971	5.256
3-Carene	0.274	ND	ND	0.094	0.038	0.611	0.022	0.151	0.040
Alpha terpinene	0.268	0.157	0.161	0.085	0.062	0.711	0.047	0.202	0.078
trans-beta-Ocimene	ND	ND	ND	0.209	0.162	0.257	0.168	0.264	0.223
Limonene	4.804	4.460	2.339	12.970	15.036	14.493	17.182	22.887	23.841
Cymene	ND	ND	ND	ND	ND	ND	ND	ND	ND
beta-Ocimene	ND	ND	ND	6.266	2.588	9.511	2.484	0.672	4.881
gamma-Terpinene	0.536	0.320	0.335	0.149	0.075	0.526	0.066	0.152	0.078
Terpinolene	0.587	0.349	0.357	0.245	0.194	15.210	0.214	0.474	0.970
Linalol	5.853	6.842	2.405	2.437	6.197	3.291	8.303	8.158	3.471
L-Fenchone	ND	ND	ND	0.188	0.292	0.181	0.451	0.574	0.382
Fenchol, exo-	ND	ND	ND	1.083	3.634	2.914	4.273	6.154	5.447
Isopulegol	ND	ND	ND	ND	ND	ND	ND	ND	ND
Camphor	ND	ND	ND	0.007	0.006	0.006	0.009	0.015	0.010
Isoborneol	ND	ND	ND	ND	ND	ND	ND	ND	ND
Menthol	ND	ND	ND	ND	ND	ND	ND	ND	ND
Borneol	0.247	ND	ND	0.359	0.627	0.556	0.569	1.119	0.863
alpha-Terpineol	1.604	1.176	0.347	1.147	2.359	2.150	2.640	4.115	2.957

Cannabis strain	BB*	AK*	WW*	HE*	SG*	LS*	GE*	FC*	AFG*
Citronellol	ND	ND	ND	0.001	0.002	ND	ND	0.030	ND
Geraniol	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pulegone	ND	ND	ND	ND	ND	ND	ND	ND	ND
Geranyl acetate	ND	ND	ND	0.014	ND	ND	ND	ND	ND
Alpha-Cedrene	ND	ND	ND	ND	ND	ND	ND	ND	ND
beta-Caryophyllene	ND	ND	ND	4.542	18.268	23.480	33.033	25.800	19.883
alpha-Humulene	7.759	7.260	3.141	2.932	4.962	5.844	7.798	6.783	5.440
cis-Nerolidol	ND	ND	ND	ND	ND	ND	ND	ND	ND
trans-Nerolidol	ND	ND	ND	0.169	1.114	1.181	2.272	1.322	1.120
Guaiol	ND	ND	ND	1.154	1.303	ND	ND	ND	0.181
Cedrol	ND	ND	ND	ND	ND	ND	ND	ND	ND
alpha-Bisabolol	ND	ND	ND	0.571	1.019	0.183	0.243	0.604	0.680
beta-Eudesmol	ND	ND	ND	1.371	1.620	0.099	0.105	0.146	0.315
Phytol	ND	ND	ND	0.014	ND	ND	ND	ND	ND

**Cannabis species: BB (Big Bud), AK (AK-47), WW (White Widow), HE (Herijuana), SG (Strawberry Glue), LS (La S.A.G.E), GE (Gelato), FC (French Cookies), AFG (Afghan Berry), ND – Not Detected*

Table 6. Determination of major terpenoids in cannabis extracts obtained from different varieties of cannabis flower

Cannabis strain	WW*		
Terpene	**Terpenes (%) in dry cannabis flowers**	**Terpenes (%) in Decarboxylated oil**	**Terpenes (%) in Distillated oil**
alpha-Pinene	15.287	4.089	0.69
beta-Myrcene	68.887	15.225	1.90
beta-Pinene	6.438	2.860	0.11
Cannabis strain	**BB***		
Terpene	**Terpenes (%) in dry cannabis flowers**	**Terpenes (%) in Decarboxylated oil**	**Terpenes (%) in Distillated oil**
alpha-Pinene	10.997	1.27	0.73
beta-Myrcene	62.998	42.91	3.76
Limonene	4.804	3.70	1.07

**Decarboxylated and Distillated oil obtained from cannabis strain WW (White Widow) and BB (Big Bud)*

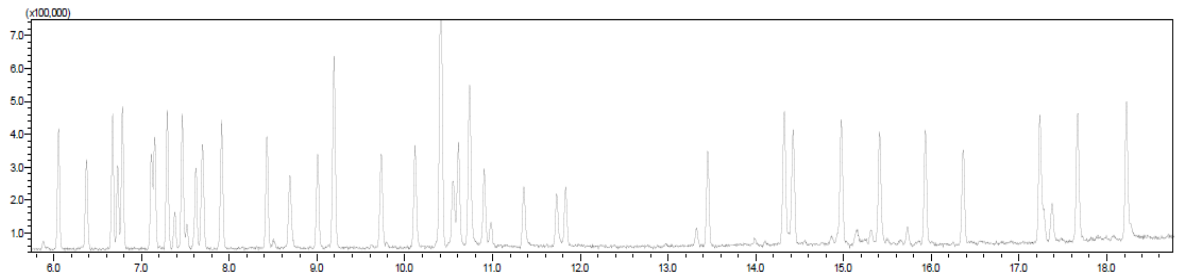

Fig. 2. Typical chromatogram for chromatographic separation of terpenes in standard solution

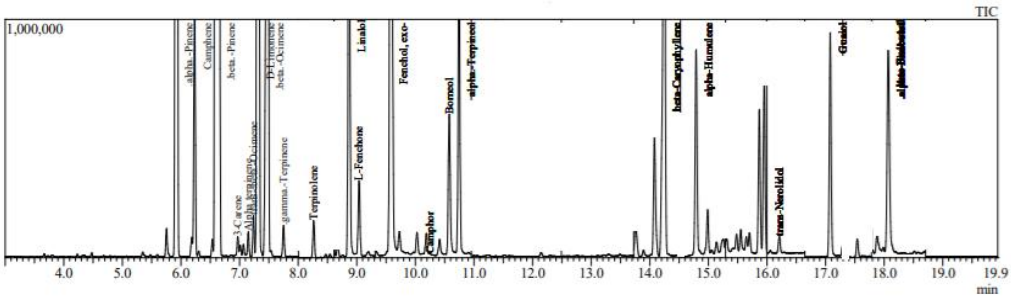

Fig. 3. Typical chromatogram for chromatographic separation of terpenes in cannabis flower

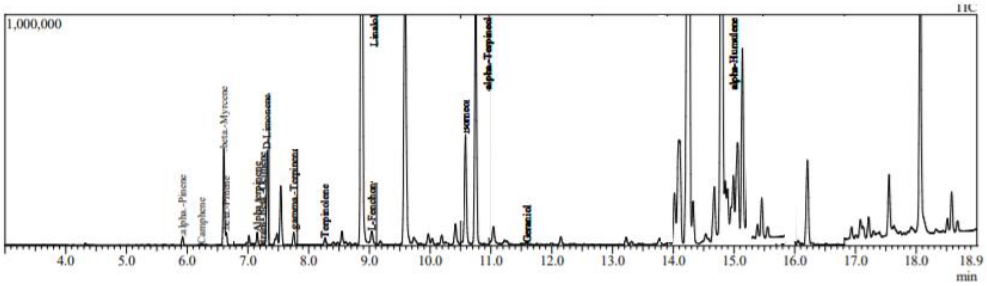

Fig. 4. Typical chromatogram for chromatographic separation of terpenes in cannabis extract

3.2 Determination of Terpenoid Profile in Cannabis Dry Flowers and Cannabis Extracts

Results from determination of terpenoid profile on nine different varieties of cannabis plant are shown in Table 5.

Results from determination of three major terpenoids in cannabis extracts (two decarboxylated and two distillated oils) obtained from two different varieties of cannabis dry flowers compared with quantity (in %) of terpenes in the cannabis dry flowers used for process of extraction are shown in Table 6.

Typical chromatogram for chromatographic separation of all terpenes in standard solution is shown in Fig. 2.

Typical chromatogram for chromatographic separation of all terpenes in cannabis flower is shown in Fig. 3.

Typical chromatogram for chromatographic separation of all terpenes in cannabis extract is shown in Fig. 4.

4. DISSCUSION

Terpenes, which are the basic ingredients of essential oils in many plants have been used for thousands of years for different therapeutic purposes. Studies in animal models and humans have identified analgesics, antimicrobials, anti-inflammatory and similar therapeutic properties. The main focus of researchers for the therapeutic purposes of cannabis-based medicines have been cannabinoids primarily Δ9-tetrahydrocannabinol (THC), while terpenes and potential interactions between terpenes and cannabinoids has barely been studied at all when the cannabis-based medicines are consumed for medical purposes [36].

The hypothesized synergistic interactions between different cannabinoids and terpenes to obtain unique pharmacological effects have been investigated in several preclinical and some clinical studies. There is skepticism in the literature and remains unclear with insufficient evidence from preclinical studies whether terpenes can act synergistic with cannabinoids [37-39]. If terpenes can be shown to modulate cannabinoid activity, it could provide a powerful tool to improve cannabinoid therapy.

Recently studies have been conducted to evaluate the functional and modulatory actions of various terpenes in vivo and in vitro, both alone and in combination with an established cannabinoid agonist. The results of this studies establish direct interaction between cannabinoids

and terpenes demonstrating that terpenes can selectively modulate pharmacological agonist activity of cannabinoids. This study is the first that shows that terpenes and cannabinoids can produce an additive effect when combined [36]. The mechanisms of synergistic action between terpenes and cannabinoids at the molecular level is still unknown. Two alternatives are (1) direct modulation of membrane shifting CB1 receptors activation and (2) terpene modulation of endocannabinoid synthesis or degradation, which results in CB1 receptors activation. But the notable aspect of this study was the generally high concentrations of terpenes needed to see activation [36].

In our case, we conducted tests for determination of terpenes in cannabis dry flowers and extracts. Since there is no monograph in the European Pharmacopeia (Ph.Eur.) for quality testing of cannabis flower and extracts, currently a revised monograph for cannabis flower (cannabis floss) and cannabis extracts, published in the German Pharmacopoeia in 2018 (3) and 2020, by the Federal Institute for Drugs and Medical Devices (BfArM) has instructed the obligatory procedure for quality testing of cannabis flowers in the European Union [40]. Following these monographs, the purification process of the cannabis crude extract is necessary to achieve a final concentration of THC of more than 95% in the final active pharmaceutical ingredient (API) as it is note in the monograph. With the analysis performed we have shown that the purification process removes terpenes from the final extracts.

The percentage of major terpen beta-Myrcene which is the smallest terpene with proved antipsychotic, antioxidant, analgesic, anti-inflammatory, sedative, muscle relaxant and anticancer effects in the starting material decreased from 68 to 15% in decarboxylated and, 1.9% in distillated cannabis oil after purification.

The percentage of second major terpene alpha-Pinene which has proven antibacterial, anti-inflammatory, broncho dilatory, antiseptic and gastroprotective pharmacological effects in the starting material decreased from 15 to 5% in decarboxylated and, 0.7% in distillated cannabis oil after purification.

Considering that generally high concentrations of terpenes are needed to see cannabinoid CB1 receptors activation the question that arise is whether the process of purification to obtain an API with a higher concentration of cannabinoids is justified.

5. CONCLUSION

The main carriers of pharmacological effects in cannabis flowers or extracts are cannabinoids. Terpenes itself have a wide range of pharmacological actions and act synergistically with cannabinoids in exhibiting a pharmacological effect. At the same time terpenes are fingerprint or a specificity that is characteristic of each variety, which is very important for standardization of the cannabis-based extracts. Following the German monograph for cannabis extracts the purification process is necessary to achieve a final concentration of THC of more than 95% in the final active pharmaceutical ingredient. With the analysis performed we have shown that the purification process removes terpenes from the final extracts. The percentage of major terpen beta-Myrcene which has proven antipsychotic, antioxidant, analgesic, anti-inflammatory, sedative, muscle relaxant and anticancer effects decreased from 68% in dry flower to 15% in decarboxylated and, 1.9% in distillated cannabis oil after purification. The percentage of second major terpene alpha-Pinene which has proven antibacterial, anti-inflammatory, broncho dilatory, antiseptic and gastroprotective pharmacological effects decreased from 15% in dry flower to 5% in decarboxylated and, 0.7% in distillated cannabis oil after purification. The question that arises is connected to the pharmacological effect on cannabis-based medicines obtained from cannabis active pharmaceutical ingredients in which terpenes have been removed.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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